11 Chitin Metabolism in Insects

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11.1. Introduction

"Chitin Metabolism in Insects" was the title of one of the chapters in the original edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* series published in 1985 (Kramer *et al.*, 1985). Since that time substantial progress in gaining an understanding of this topic has occurred, primarily through the application of techniques of molecular genetics and biotechnology to assorted studies on insect chitin metabolism. Several other reviews have also been published, which have reported on some of the advances that have taken place (Kramer and Koga, 1986; Cohen, 1987, 2001; Koga *et al.*, 1999; Fukamizo, 2000). Thus, in this chapter we will highlight some of the more important findings since 1985, with an emphasis on results obtained from studies conducted on the two enzymes primarily responsible for chitin synthesis and degradation, namely chitin synthase (CHS) and chitinase (CHI).

11.2. Chitin Structure and Occurrence

Chitin is widely distributed in animals and represents the skeletal polysaccharide of several phyla such as the Arthropoda, Annelida, Mollusca, and Coelenterata. In several groups of fungi, chitin replaces cellulose as the structural polysaccharide. In insects, it is found in the body wall, gut lining, cuticle, salivary glands, trachea, mouth parts, and muscle attachment points.

In the course of evolution, insects have made excellent use of the rigidity and chemical stability of the polymeric chitin to assemble extracellular structures such as the cuticle (exoskeleton) and gut lining (peritrophic membrane (PM)), both of which enable insects to be protected from the environment while allowing growth, mobility, respiration, and communication. Several genes and gene products are involved in chitin metabolism in insects. In general there are two primary extracellular structures in

which chitin deposition occurs. Those are the cuticle and the PM where both synthesis and degradation of chitin take place at different developmental stages.

Chitin is the major polysaccharide present in insects and many other invertebrates and several microbes. Structurally, it is the simplest of the glycosaminoglycans, being a β (1→4) linked linear homopolymer of *N*-acetylglucosamine (GlcNAc, $(C_8H_{13}O_5N)_{n\gg1}$). It is usually synthesized as the old endocuticle and PM are resorbed and the digested materials are recycled. Because of the intractable nature of insect sclerotized structures such as cuticle, there was very little quantitative data available about chemical composition until recently when solid-state nuclear magnetic resonance (NMR) was utilized for analyses. The cuticle and PM are composed primarily of a mixture of protein and chitin, with the former usually predominating (Kramer *et al.*, 1995). Chitin contents vary substantially depending on the type of cuticle. For example, in the sclerotized puparial cuticle from the housefly, *Musca domestica*, the chitin content is approximately 45% of the wet weight, whereas in the mineralized puparial cuticle of the face fly, *Musca autumnalis*, the chitin content is only about 19% (Roseland *et al.*, 1985; Kramer *et al.*, 1988). In larval, pupal, and adult cuticles of the tobacco hornworm, *Manduca sexta*, the chitin content is approximately 14%, 25%, and 7%, respectively (Kramer *et al.*, 1995). In newly ecdysed pupal cuticle, there is only about 2% chitin prior to sclerotization, but that amount increases more than 10-fold after sclerotization. When cuticular protein and chitin are mixed, they form a matrix in which the components of lower abundance, such as water, catechols, lipids, and minerals, are interspersed. The PM of the tobacco hornworm is made up primarily of protein (60%) and chitin (40%) (Kramer *et al.*, 1995). Although primarily composed of poly-GlcNAc, chitin also can contain a small percentage of unsubstituted (or *N*-deacetylated) glucosamine (GlcN) residues (Fukamizo *et al.*, 1986). When the epidermal and gut cells synthesize and secrete a particular form of chitin consisting of antiparallel chains, α-chitin, the chains are formed into sheets. As layers are added, the sheets become cross-oriented to one another, which can contribute to the formation of an extremely strong plywood-like material. The origin of proteins in the cuticle is unknown, but some hemolymph proteins are deposited in cuticle. Thus, apparently the epidermal cells do not need to supply all of the component parts of the exoskeleton. The cells lining the gut produce some of the PM-associated proteins and these proteins are referred to as the peritrophins (Tellam *et al.*, 1999; Wang and Granados, 2000a; Bolognesi *et al.*, 2001; Eisemann *et al.*, 2001). Analysis of expressed sequence tags in the cat flea, *Ctenocephalides felis*, demonstrated that some peritrophins are produced exclusively by hindgut and Malpighian tubule tissues (Gaines *et al.*, 2002).

The last step in cuticle formation, tanning, involves modification of the free amino acid tyrosine that is sequestered as a conjugate with glucose in the hemolymph. Tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA), and then decarboxylated to 3,4-dihydroxyphenethylamine (dopamine) (Hopkins and Kramer, 1992). Dopamine is *N*-acylated with acetate or β-alanine in the epidermal cells and sequestered in the hemolymph as conjugates with glucose, sulfate, or another hydrophilic compound. The *N*-acylated dopamine conjugates then are delivered through pore canals to the epicuticle where the conjugates are hydrolyzed and then converted by phenoloxidases to very highly reactive quinones and quinone methides. These transient compounds then cross-link proteins to form tanned proteins in a process known as sclerotization. These cross-linked proteins and chitin make up most of the exocuticle. Chitin chains also may become cross-linked with cuticular proteins, but the evidence for that is not definitive.

Chitin oligosaccharides that are produced during degradation of chitin by chitinases appear to play an important role in insect immunity towards microorganisms. The basic immune strategy against microbial infection in insects appears to be similar to the strategy used by plants against fungal infection. These oligosaccharides are known to activate chitinase genes in plants, which are actively involved in the plant defense response against fungal infection (Nichols *et al.*, 1980). In the silkworm, *Bombyx mori*, chitin oligomers trigger expression of three different antibacterial proteins – cecropin, attacin, and lebocin – in the fat body and hemocytes (Furukawa *et al.*, 1999).

11.3. Chitin Synthesis

Relatively little additional biochemical data on the enzymes of the chitin biosynthetic pathway have been generated since the previous review was published (Kramer *et al.*, 1985). The paucity of information concerning the biochemical properties of these enzymes is due to the inability to obtain soluble preparations of CHSs and the instability of the glutamine-fructose-6-phosphate aminotransferase (GFAT), the enzyme that provides

the GlcN precursor of the chitin biosynthetic pathway. However, CHSs have been identified in a variety of organisms, including nematodes, fungi, and insects. Amino acid sequence similarities have been the principal tools used for identifying CHSs, which form a subfamily within a larger group (family GT2) of the glycosyltransferases that catalyze the transfer of a sugar moiety from an activated sugar donor onto saccharide or nonsaccharide acceptors (Coutinho and Henrissat, 1999; Coutinho *et al.*, 2003; CAZY, 2004). During the past 3 years, there has been a sudden increase in research in the area of chitin synthesis. The impetus for this enhanced interest has come predominantly from cloning of genes for the two key enzymes of the pathway, GFAT and CHS, from insects.

CHS has not been an easy enzyme to assay, which has made its study rather difficult. Traditionally, CHS activity was measured by a radioactive assay using [^{14}C]UDP-GlcNAc as the substrate followed by quantification of insoluble ^{14}C-labeled chitin after acid precipitation. Recently, however, a high throughput nonradioactive assay has been developed (Lucero *et al.*, 2002). The procedure involves binding of synthesized chitin to a wheat germ agglutinin (WGA)-coated surface followed by detection of the polymer with a horseradish peroxidase–WGA conjugate. This nonradioactive assay should facilitate greater progress in CHS studies in the future.

11.3.1. Precursors of the Chitin Biosynthetic Pathway

Early studies on chitin synthesis using whole insects or isolated tissues demonstrated that in addition to whole animals, a variety of tissues including larval and pupal epidermis, abdomen, integument, gut, imaginal discs, leg regenerates, hypodermis, and oocytes were capable of synthesizing chitin (review: Kramer *et al.*, 1985). An assortment of compounds, including glycogen, glucose, glucosamine, fructose, and GlcNAc could serve as biosynthetic precursors of chitin in these tissues. These early studies also identified several compounds that inhibited the pathway. This list includes substrate analogs such as tunicamycin, polyoxin D, nikkomycin, and uridine diphosphate (UDP), as well as several compounds belonging to the benzoylphenylurea class of insect growth regulators whose exact mode of action has not yet been established. Results of these studies also indicated that ecdysone may influence chitin synthesis either directly or indirectly. However, the details of such a regulation remain unclear.

11.3.2. Sites of Chitin Biosynthesis

The epidermis and the midgut are two major tissues where chitin synthesis occurs in insects. Epidermal cells are responsible for the deposition of new cuticle during each molt and the midgut cells are generally associated with the formation of the PM during feeding. Chitin is associated with other tissues as well, including the foregut, hindgut, trachea, wing hinges, salivary gland, and mouth parts of adults and/or larvae (Wilson and Cryan, 1997). In general, it is assumed that the cells closest to the site where chitin is found are responsible for its biosynthesis. However, this interpretation is complicated by the fact that assembly of chitin microfibrils occurs in the extracellular space and is influenced by the presence or absence of associated proteins. This is particularly true in the gut where some cells around the cardia may be contributing to chitin synthesis and secretion, whereas other cells in different parts of the gut may be responsible for synthesis of PM-associated proteins (Wang and Granados, 2000a). Visible PM may appear at sites remote from the original site of synthesis of either chitin or PM proteins.

11.3.3. Light and Electron Microscopic Studies of Peritrophic Membrane Synthesis

The most detailed picture of chitin synthesis and its association with proteins to form the composite PM has emerged from observations using light microscopy as well as transmission and scanning electron microscopy (SEM) of PM synthesis in the three lepidopteran insects, *Ostrinia nubilalis* (European corn borer), *Trichoplusia ni* (cabbage looper), and *M. sexta* (Harper and Hopkins, 1997; Harper *et al.*, 1998; Harper and Granados, 1999; Wang and Granados, 2000a; Hopkins and Harper, 2001). The presence of chitin in nascent PM can be followed by staining with gold-labeled WGA, which binds to GlcNAc residues in chitin and glycoproteins. This method was used to show that chitin-containing fibrous material appears first at the tips of the microvilli of the midgut epithelial cells of *O. nubilalis* just past the stomadeal valves and is rapidly assimilated into a thin PM surrounding the food bolus (Harper and Hopkins, 1997). The PM becomes thicker and multilayered in the middle and posterior regions of the mesenteron. The orthogonal lattice of chitin meshwork is slightly larger than the diameter of the microvilli. SEM and light microscopic studies revealed that the PM delaminates from the tips of the microvilli. This observation suggests that microvilli serve as sites and possibly as templates for the organization of the PM by laying down a matrix of chitin microfibrils onto

which some PM proteins are deposited. A similar pattern of delamination of PM containing both chitin and intestinal mucins was demonstrated in larvae of *T. ni* (Harper and Granados, 1999; Wang and Granados, 2000a).

Incorporating WGA into the diet can interrupt formation of the PM. WGA-fed *O. nubilalis* larvae had an unorganized PM, which was multilayered and thicker than the normal PM (Hopkins and Harper, 2001). WGA was actually associated with the PM as well as with the microvillar surface as revealed by immunostaining with antibodies specific for WGA. Because there was very little WGA within the epithelial cells, the action of WGA appears to be extracellular. Presumably, WGA interferes with the formation of the organized chitin network and/or the association of PM proteins with the chitin network, leading to a reduced protein association with the PM (Harper *et al.*, 1998). There was also extensive disintegration of the microvilli and the appearance of dark inclusion bodies as well as apparent microvillar fragments within the thickened multilayered PM. Insects such as *M. sexta*, which secrete multiple and thickened PMs that are somewhat randomly organized, tolerated WGA better and sequestered large amounts of WGA within the multilayered PM (Hopkins and Harper, 2001).

11.3.4. *In Situ* Hybridization and Immunological Studies

In situ hybridizations with a DNA probe for the catalytic domain of a CHS revealed that high levels of transcripts for this gene are present in apical regions of the columnar cells of the anterior midgut of *M. sexta* larvae (Zimoch and Merzendorfer, 2002). Lesser amounts of CHS transcripts were detected in the posterior midgut. An antibody to the catalytic domain of *M. sexta* CHS also detected the enzyme in midgut brush border membranes at the extreme apical ends of microvilli, suggestive of some special compartment or possibly apical membrane-associated vesicles. Staining was also seen in apical membranes of tracheal and salivary gland cells. Materials reacting with CHS antibody also were detected underneath the epidermal cuticle, even though it could not be specifically assigned to the apical membrane of epidermal cells due to loss of structural integrity of these cells during cryosectioning. These *in situ* hybridization and immunochemical studies are in agreement with earlier observations about chitin synthesis in *Calpodes ethlius* (larger canna leafroller), which indicated the involvement of specialized structures called plasma membrane plaques found in apical portions of epidermal cells (Locke and Huie, 1979). Comparable electron microscope (EM) and immunological localization of CHS associated with epidermis during cuticle deposition have not been reported primarily because of technical difficulties with the handling of cuticular samples. In *Drosophila melanogaster* the chitin synthase gene (*kkv*) is expressed predominantly in developmental stages 13-14 in the embryonic ventral and dorsal epidermis, foregut and in the larval tracheal system (see the "Patterns of gene expression in *Drosophila* embryogenesis" at the Berkeley Drosophila Genome Project (BDGP)).

11.3.5. Chitin Biosynthetic Pathway

It has been assumed that the pathway of chitin biosynthesis in insects would be similar or identical to the pathway that has been worked out extensively in fungi and other microbes (**Figure 1**). This appears to be the case except for some minor details (Palli and Retnakaran, 1999). The source of the sugar residues for chitin synthesis can be traced to fat body glycogen, which is acted upon by glycogen phosphorylase. Glucose-1-P produced by this reaction is converted to trehalose, which is released into the hemolymph. Trehalose, the extracellular source of sugar in many insects, is acted upon by a trehalase, which is widely distributed in insect tissues including the epidermis and gut to yield intracellular glucose (Becker *et al.*, 1996). The conversion of glucose to fructose-6-P needed for chitin synthesis involves two glycolytic enzymes present in the cytosol. These enzymes are hexokinase and glucose-6-P isomerase, which convert glucose to fructose-6-P. From the latter, the chitin biosynthetic pathway branches off, with the first enzyme catalyzing this branch being GFAT, which might be thought of as the first committed step in amino sugar biosynthesis. The conversion of fructose-6-P to GlcNAc phosphate involves amination, acetyl transfer, and an isomerization step, which moves the phosphate from C-6 to C-1 (phosphoacetylglucoasmine mutase). The conversion of this compound to the nucleotide sugar derivative follows the standard pathway and leads to the formation of a UDP-derivative of GlcNAc, which serves as the substrate for CHS. The entire chitin biosynthetic pathway is outlined in **Figure 1**.

The involvement of dolichol-linked GlcNAc as a precursor for chitin was proposed quite some time ago (Horst, 1983), but it has received very limited experimental support (Quesada-Allue, 1982). At this point, this possibility remains unproven. Similarly, the requirement for a primer to which the

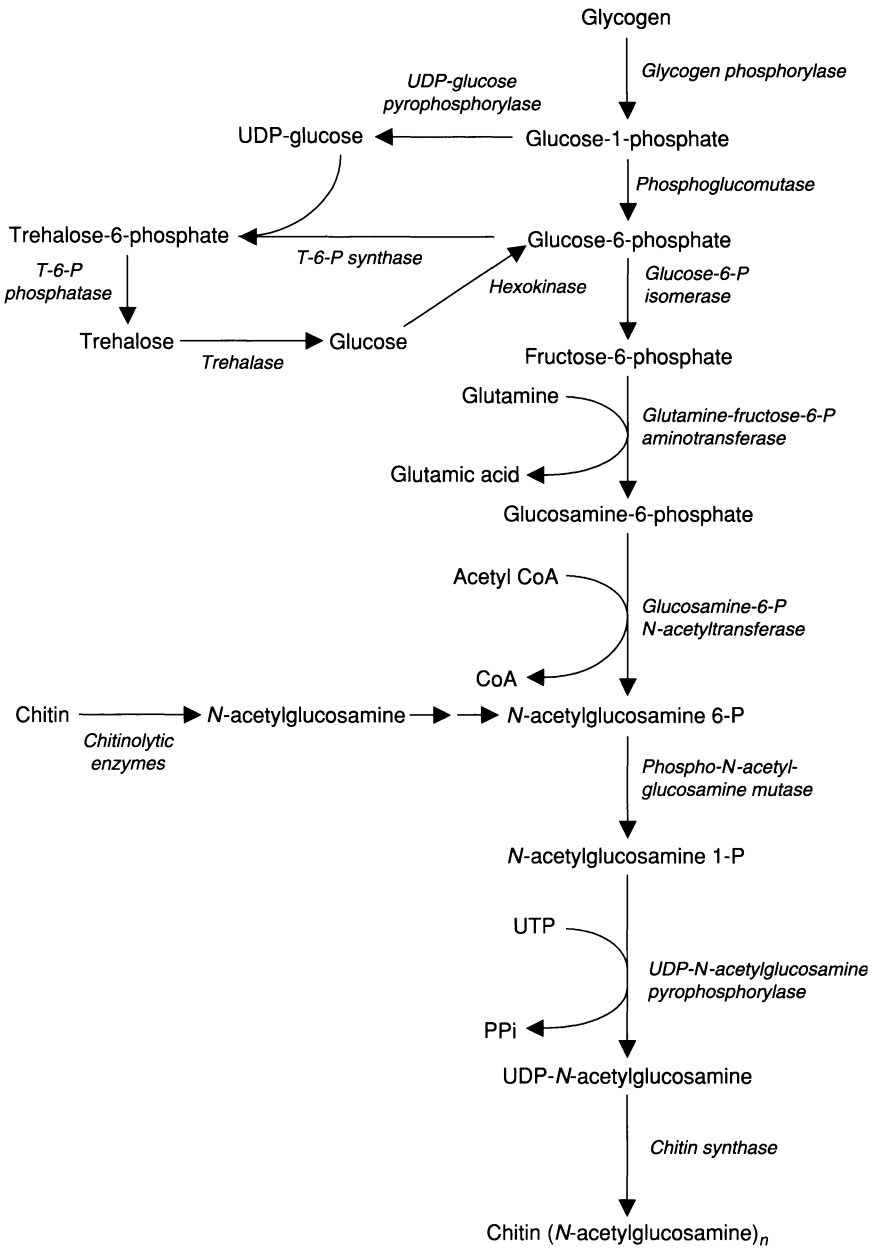


Figure 1 Biosynthetic pathway for chitin in insects starting from glycogen, trehalose, and recycled chitin.

GlcNAc residues can be transferred also remains speculative. Based on the model for glycogen biosynthesis, which requires glycogenin as the primer (Gibbons *et al.*, 2002), CHS or an associated protein may fulfill this priming function. Because each sugar residue in chitin is rotated ~180° relative to the preceding sugar, which requires CHS to accommodate a alternating "up/down" configuration, another precursor, UDP-chitobiose, has been proposed to be a disaccharide donor during biosynthesis (Chang *et al.*, 2003). However, evaluation of radiolabeled UDP-chitobiose as a CHS substrate in yeast revealed that it was not a viable one. Even at elevated concentrations, no incorporation of radioactivity above background was observed using membranous preparations of CHS from the yeast *Saccharomyces cerevisiae* (Chang *et al.*, 2003).

11.3.5.1. Key enzymes The biosynthetic pathway of chitin can be thought of as consisting of two segments. The first set of reactions leads to the formation of the amino sugar, GlcNAc, and the second set of reactions leads to the synthesis of the polymeric chitin from the amino sugar. The

rate-limiting enzyme in the first segment appears to be GFAT (also known as glucosamine-fructose-6-phosphate aminotransferase (GFAT, EC 2.6.1.16), which is found in the cytosol. The critical enzyme in the second segment is CHS (EC 2.4.1.16), which is localized in the plasma membrane. Not surprisingly, these two enzymes appear to be major sites of regulation of chitin synthesis.

11.3.5.2. Regulation of glutamine-fructose-6-phosphate aminotransferase synthesis

11.3.5.2.1. Drosophila GFAT Two genes encoding GFAT (*Gfat1* and *Gfat2*) have been identified in *Drosophila* (Adams *et al.*, 2000; Graack *et al.*, 2001). Both of these genes are on chromosome 3, but they are at different locations. Their intron–exon organizations are different as are the amino acid sequences of the encoded proteins. GFAT consists of two separate domains, an N-terminal domain that has both glutamine binding and aminotransferase motifs identified in GFATs from other sources and a C-terminal domain with both fructose-6-phosphate binding and isomerase motifs. *Gfat1* is expressed in embryos in the developing trachea and in cuticle-forming tissues including the chitinous mouth armature of the developing first instar larva. In the last larval stadium, *Gfat1* is expressed in the corpus cells of salivary glands, but this synthesis may be related to the production of the highly glycosylated Sgs glue proteins (Graack *et al.*, 2001). The major regulation of GFAT1 appears to be posttranslational. When *Gfat1* was expressed in yeast cells, the resulting enzyme was feedback inhibited by UDP-GlcNAc and was stimulated by protein kinase A. Even though it has not been demonstrated that there is a phosphorylated form of GFAT1 that is susceptible to feedback inhibition by UDP-GlcNAc, this possibility remains viable. The expression and regulation of the other GFAT isozyme (GFAT2) has not yet been reported.

11.3.5.2.2. Aedes aegypti GFAT The gene and cDNA for the mosquito *Aedes aegypti* GFAT1 have been cloned (Kato *et al.*, 2002). The mosquito gene has no introns and the promoter appears to contain sequences related to ecdysteroid response elements (EcRE) as well as E74 and Broad complex Z4 elements. E74 and Broad complex Z4 proteins are transcription factors known to be upregulated by ecdysone (Thummel, 1996). Two *Gfat1* transcripts with different sizes were observed in Northern blot analyses of RNA from adult females and their levels increased further after blood-feeding (Kato *et al.*, 2002). Since ecdysteroid titers increase following blood-feeding, it is possible that this gene is under the control of ecdysteroid either directly or indirectly. Feedback inhibition by UDP-GlcNAc has not been reported, but the *Aedes* enzyme is likely to be regulated in a manner similar to the *Drosophila* enzyme by this effector and possibly by a phosphorylation/dephosphorylation mechanism as well.

11.3.5.3. CHS gene number and organization

CHS genes from numerous fungi have been isolated and characterized (Munrow and Gow, 2001). However, the complete sequence of a cDNA clone for an insect CHS (sheep blowfly, *Lucilia cuprina*) was reported only recently (Tellam *et al.*, 2000). Since then, the sequences of several other full-length cDNAs and genes for CHSs from other insects and nematodes have been reported. The nematode CHSs were from two filarial pathogens, *Brugia malayi*, and *Dirofilaria immitis*, and the plant parasite *Meloidogyne artiellia* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). The other insect species from which CHS cDNAs have been isolated are *A. aegypti* (Ibrahim *et al.*, 2000), *M. sexta* (Zhu *et al.*, 2002) and the red flour beetle, *Tribolium castaneum* (Arakane *et al.*, 2004). DNA sequencing of polymerase chain reaction (PCR)-amplified fragments encoding a highly conserved region in the catalytic domains of insect CHSs indicates a high degree of sequence conservation (Tellam *et al.*, 2000). In addition, a search of the databases in light of the sequence data from these cDNAs has allowed identification of open reading frames (ORFs) from CHS genes from *Drosophila*, *Anopheles, Aedes* and the nematode *Caenorhabditis elegans* (Tellam *et al.*, 2000; Gagou *et al.*, 2002; Arakane *et al.*, 2004). **Table 1** lists the properties of insect CHSs encoded by these genes/cDNAs. Insect species typically have two genes for CHSs. Among the nematodes, the *C. elegans* genome contains two CHS genes, but so far there is evidence for only one gene in the plant parasitic nematode *M. artiellia*, and in the filarial nematodes *B. malayi* and *D. immitis* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). Fungi, on the other hand, exhibit a wide range in the number of genes for CHS (Munrow and Gow, 2001).

The two *Tribolium* CHS genes, *TcCHS1* and *TcCHS2*, have ten and eight exons, respectively (Arakane *et al.*, 2004). The organizations of the two genes in *Tribolium* are quite different, with some introns occurring in identical positions in both genes, whereas others are at variable positions. The introns ranged in length from 46 bp to more than 3000 bp. The most interesting difference between the two genes was the presence of two

Table 1 Properties of insect chitin synthases and their genes

Species	Number of amino acids	Expressed in	Alt. Exon	Coiled-coil	CHS class	GI no.	Reference
Lucilia cuprina	1592	Epidermis	Yes[a]	Yes	A	9963823	Tellam *et al.* (2000)
Drosophila melanogaster	1615	Epidermis/gut/tracheal	Yes	Yes	A	24644218	Adams *et al.* (2000);
	1674		Yes	Yes	A	24644220	Fly base – http://www.flybase.bio.indiana.edu; Berkeley Drosophila genome project (Drosophila EST database) – http://www.fruitfly.org
	1416	ND	No	No	B	24668460	
Anopheles gambiae	1578		Yes	Yes	A		
	1583		No	No	B		
Aedes aegypti	1564	Midgut	No	No	B	22773456	Ibrahim *et al.* (2000)
Tribolium castaneum	1558	ND	Yes	Yes	A		
	1558	ND	Yes	Yes	A		Arakane *et al.* (2004)
	1464	ND	No	No	B		
Manduca sexta	1563	Epidermis/gut	Yes	Yes	A	24762312	Zhu *et al.* (2002)
	1563	Epidermis/gut	Yes	Yes	A		H. Merzendorfer (unpublished data)
	1524	Gut	No	No	B		D. Hogenkamp *et al.* (unpublished data)

[a]Predicted.
ND, not determined.

nonidentical copies of exon 8 (named 8a and 8b) in *TcCHS1*, whereas *TcCHS2* has only one copy of this region as a part of exon 6. An analysis of genomic sequences from the *D. melanogaster* and *Anopheles gambiae* genome projects, partial sequencing of cDNAs available as separate sequence files submitted to GenBank, and "TBLASTN" queries were used to determine the organization of CHS genes in these insects (**Figure 2**). These analyses revealed that the sequences and organization of CHS genes of *D. melanogaster* (Tellam *et al.*, 2000) and *A. gambiae* were similar to those of *TcCHS1* and *TcCHS2* (Arakane *et al.*, 2004). One major difference between the two exons that are alternately spliced is that all of the B forms code for segments that have a site for *N*-linked glycosylation just before the transmembrane helix, whereas none of the A forms do. The physiological significance of alternate exon usage and potential glycosylation in *CHS* expression is unknown even though it is clear that there is developmental regulation of alternate exon usage (see Section 11.3.5.6).

11.3.5.4. Modular structure of chitin synthases CHSs are members of family GT2 of the glycosyltransferases (Coutinho *et al.*, 2003), which generally utilize a mechanism where inversion of the anomeric configuration of the sugar donor occurs. The protein fold (termed GT-A) for this family is considered to be two associated β/α/β domains that form a continuous central sheet of at least eight β-strands. The GT-A enzymes share a common ribose/metal ion-coordinating motif (termed DxD motif) as well as another carboxylate residue that acts as a catalytic base. The general organization of CHSs has been deduced from a comparison of amino acid sequences of these enzymes from several insects, nematodes and yeasts (Zhu *et al.*, 2002; Arakane *et al.*, 2004). These enzymes have three distinguishable domains: an N-terminal domain with moderate sequence conservation among different species and containing several transmembrane segments, a middle catalytic domain that is highly conserved even among CHSs from different kingdoms, and a C-terminal module with multiple transmembrane segments (**Figure 3**). The catalytic domain contains several stretches of highly conserved amino acid sequences including the following: CATMWHXT at the beginning of the catalytic domain, FEYAIGHW and VQYDDQGEDRW in the middle of the catalytic domain, and the presumed catalytic site, EFYNQRRRW, at the end of the catalytic domain. While the transmembrane segments in the N-terminal domain show different patterns among different insect species, the transmembrane segments in the C-terminal domain are remarkably conserved both with respect to their location and the spacing between adjacent transmembrane segments. Particularly striking is the fact that five such transmembrane segments are found in a cluster immediately following the catalytic domain and two more segments are located closer to the

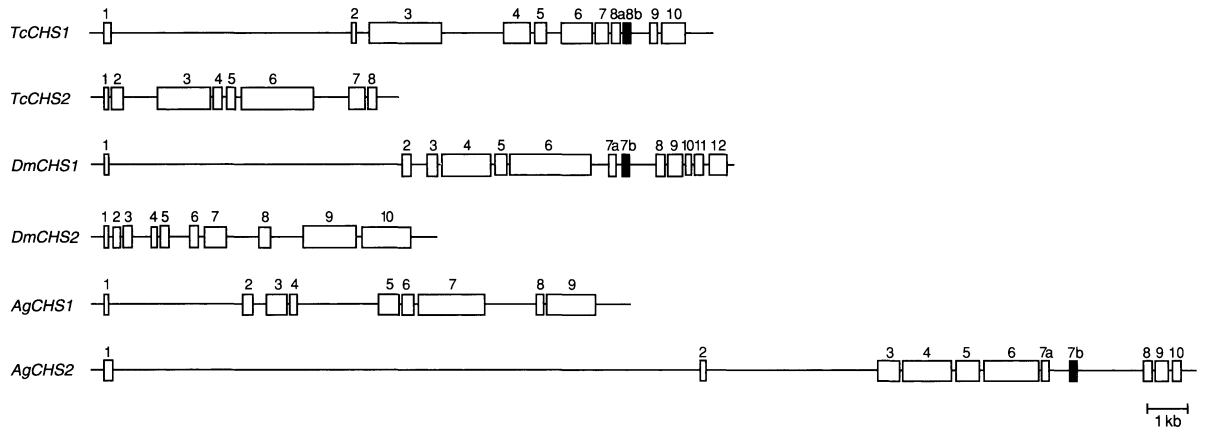


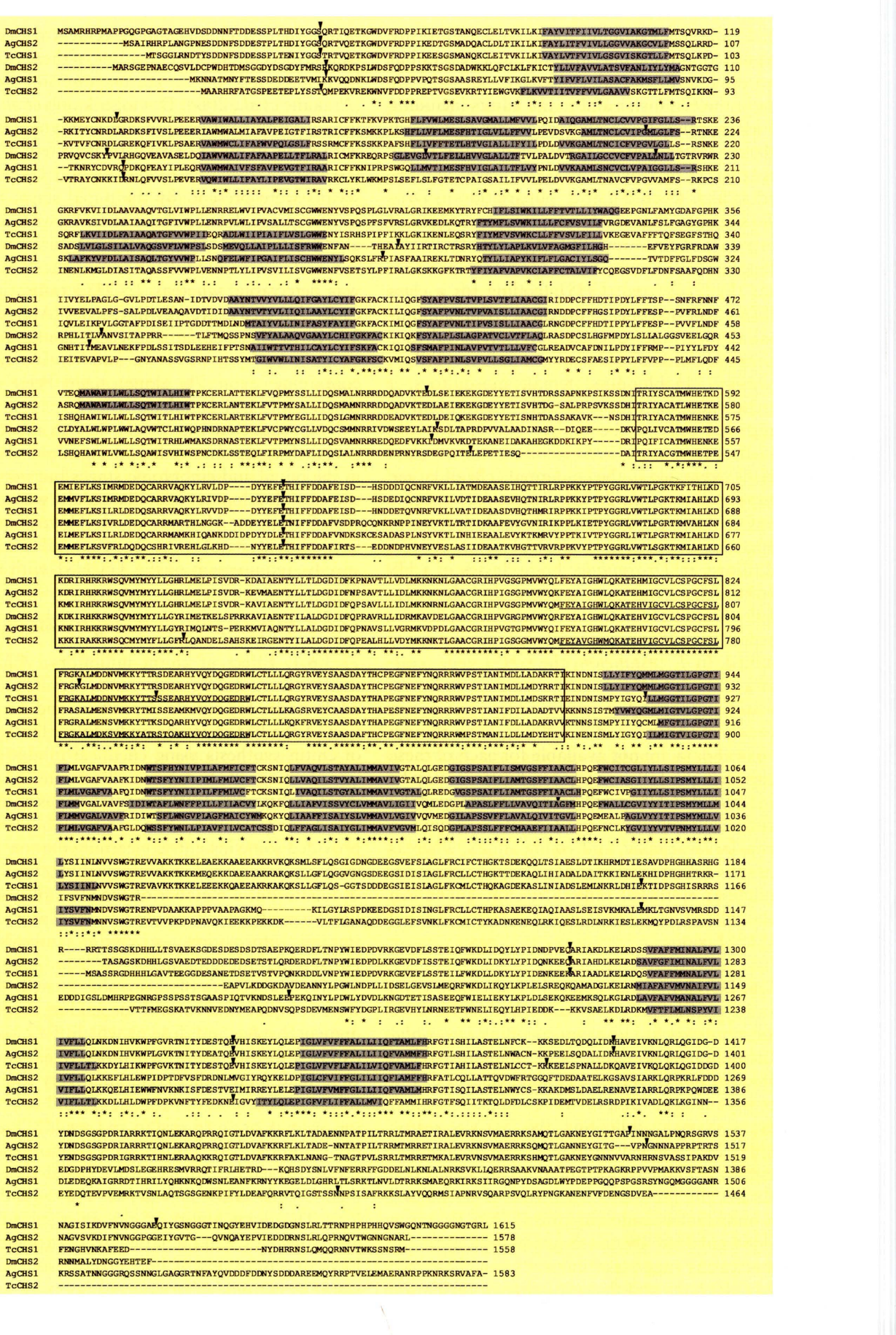
Figure 2 Schematic diagram of the organization of the *TcCHS1*, *TcCHS2*, *DmCHS1*, *DmCHS2*, *AgCHS1*, and *AgCHS2* genes. Boxes indicate exons. Lines indicate introns. The second of the two alternative exons (8b) of *TcCHS1*, *DmCHS1 (7b)*, and *AgCHS2 (6b)* are indicated as closed boxes. About 9 kb of the *TcCHS1* and *TcCHS2* gDNA sequences were compared to their respective cDNA sequences to define the exons and introns. The exon–intron organization of the other four CHS genes was deduced partially from comparisons of available cDNA and genomic sequences. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, **2004**. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol. 34*, 291–304.)

C-terminus. The 5-transmembrane cluster, known as 5-TMS, has been suggested to be involved in the extrusion of the polymerized chitin chains across the plasma membrane to the exterior of the cell as proposed for extrusion of cellulose (Richmond, 2000).

The CHSs of insects characterized so far can be broadly grouped into two classes, A and B, based on amino acid sequence identities. The class A proteins were predicted to have a coiled-coil region immediately following the 5-TMS region (Zhu *et al.*, 2002; Arakane *et al.*, 2004). Also, all of the genes encoding the class A CHSs have two alternate exons (corresponding to alternate exon 7 of *D. melanogaster*, exon 8 of *T. castaneum*, exon 6 of *A. gambiae*, and an unnumbered exon of *M. sexta* CHS-A gene) (see **Table 1**). The alternate exons are located on the C-terminal side of the 5-TMS region and encode the next transmembrane segment and flanking sequences. The alternate exon-encoded regions of the CHS proteins differ in sequence by as much as 30% and most of these differences are in the regions flanking the transmembrane segment. This finding suggests that the proteins may differ in their ability to interact with cytosolic or extracellular proteins, which might regulate chitin synthesis and/or transport. An attractive hypothesis is that these flanking sequences may influence the plasma membrane location of a CHS by interacting with cytoskeletal elements or perhaps by generation of extracellular vesicles involved in chitin assembly.

11.3.5.5. Regulation of chitin synthase gene expression The two insect genes encoding CHSs appear to have different patterns of expression during development. The high degree of sequence identity of the catalytic domains and the absence of antibodies capable of discriminating between the two isoforms have complicated the interpretation of experimental data to some extent. In some cases, the technical difficulties associated with isolation of specific tissues free of other contaminating tissues have precluded unambiguous assignment of tissue specificity of expression. Nonetheless, the following conclusions can be reached from the analyses of expression of CHS genes in several insect species. CHS genes are expressed at all stages of insect growth including embryonic, larval, pupal, and adult stages. *CHS1* genes (coding for class A CHS proteins) are expressed over a wider range of developmental stages (Tellam *et al.*, 2000; Zhu *et al.*,

Figure 3 Alignment of deduced amino acid sequences of TcCHS1, TcCHS2, DmCHS1, DmCHS2, AgCHS1, and AgCHS2 using ClustalW software. Transmembrane regions predicted using TMHMM software (v. 2.0) are shaded. Shaded arrowheads indicate the positions in the protein sequences of TcCHS1 and TcCHS2 where coding regions are interrupted by introns. Intron 1 of *TcCHS1* lies in the 5′-UTR region two nucleotides 5′ of the translation start site and is not indicated in this figure. The putative catalytic domains are boxed. Symbols below the aligned amino acid sequences indicate identical (*), highly conserved (:), and conserved residues (.). The regions in TcCHS1 and TcCHS2 corresponding to the PCR probe made from two degenerate primers representing two highly conserved sequences in CHSs are underlined. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, **2004**. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol. 34*, 291–304.)



2002). *CHS2* genes (coding for class B CHSs) are not expressed in the embryonic or pupal stages but are expressed in the larval stages, especially during feeding in the last instar and in the adults including blood-fed mosquitoes (Ibrahim *et al.*, 2000; Zimoch and Merzendorfer, 2002; Arakane *et al.*, 2004). The finding that both classes of CHS genes are expressed at high levels 3 h after pupariation in *Drosophila* suggests that both enzymes are required for postpuparial development (Gagou *et al.*, 2002).

CHS genes also show tissue-specific expression patterns. In *L. cuprina*, *CHS1* (coding for a class A CHS) is expressed only in the carcass (larva minus internal tissues) and trachea but not in salivary gland, crop, cardia, midgut or hindgut (Tellam *et al.*, 2000). In blood-fed female mosquitoes, a CHS gene encoding a class B CHS is expressed in the epithelial cells of the midgut (Ibrahim *et al.*, 2000). In *M. sexta*, *CHS1* (coding for a class A CHS) is expressed in the epidermal cells of larvae and pupae (Zhu *et al.*, 2002). Transcripts specific for class B CHS were detected only in the gut tissue (D. Hogenkamp *et al.*, unpublished data). As discussed above, in *Drosophila,* both classes of CHS genes were shown to be upregulated after the ecdysone pulse had ceased in the last larval instar, but the tissue specificity of expression of each gene was not determined. In *T. castaneum*, the *CHS1* gene (coding for a class A CHS) was expressed in embryos, larvae and pupae, and in young adults, but not in mature adults (Arakane *et al.*, 2004). Even though unequivocal data are not available for each of these insect species, the following generalizations may be made. Class A CHS proteins are synthesized by epidermal cells when cuticle deposition occurs in embryos, larvae, pupae, and young adults, whereas the class B CHS proteins are expressed by the midgut columnar epithelial cells facing the gut lumen in the larval and adult stages and is probably limited to feeding stages.

11.3.5.6. Developmental control of alternate exon usage Insect CHS genes characterized so far have eight or more exons. The genes encoding *Drosophila*, *Anopheles*, *Tribolium*, and *Manduca* class A CHSs, but not the genes encoding class B CHSs, have two alternate exons, each encoding a 59 amino acid long segment following the 5-TMS region (**Table 1**). This segment contains a 20 amino acid long transmembrane region and flanking sequences. In addition, the presence of a predicted coiled-coil region immediately following the 5-TMS region in the CHSs encoded by those genes that have the alternate exons suggests a link between these two structural features and the possibility of regulation of alternate exon usage. In agreement with this idea, transcripts containing either one of these exons have been detected in *T. castaneum* and *M. sexta* (Arakane *et al.*, 2004; D. Hogenkamp *et al.*, unpublished data). In *T. castaneum* embryos, transcripts with either exon 8a or 8b were detected, whereas in last instar larvae and prepupae, only exon 8a transcripts were present. By the pupal stage, however, transcripts with exon 8a or exon 8b were abundant along with trace amounts of a transcript with both exons. In mature adults, none of these transcripts was detectable, whereas *TcCHS2* transcripts were easily detected especially in females (Arakane *et al.*, 2004). In *Drosophila*, transcripts containing either exon 7a or both exons 7a and 7b (but not those containing exon 7b alone) have been reported (Drosophila EST Database).

It appears that the TcCHS1 with the exon 8b-encoded segment is needed during cuticle deposition in the pupal and embryonic stages but not at other stages of development. Similar results were observed with fifth instar *M. sexta* larvae (Hogenkamp *et al.*, unpublished data). The biochemical basis for a specific requirement of the TcCHS1 with the exon 8b-encoded segment is unknown.

11.3.6. Chitin Synthesis during Development

11.3.6.1. Effect of chitin inhibitors Chitin synthesis occurs during embryonic, larval, pupal, and adult stages for cuticle deposition and for production of the PM in larvae and adults. The inhibition of chitin synthesis using chemical inhibitors or by introduction of mutations affects insect development at different developmental stages and to varying degrees. Studies with "chitin inhibitors" have provided some insights concerning the role of chitin in development and its biological function. The use of lufenuron, a member of the class of insecticides known as benzoylphenylureas, has provided substantial information on chitin synthesis during *Drosophila* development (Wilson and Cryan, 1997). The effects of this insect growth regulator were complex and variable depending on the developmental stage and dose at which the insects were exposed to this agent. When newly hatched larvae were reared on a diet containing very low concentrations of lufenuron, the larvae did not die until the second or third instar and usually pupariated even though the pupae were abnormally compressed. Pharate adults either failed to eclose or died shortly after emergence and had deformed legs. The flight ability of the emerged adults was also affected when the larvae were exposed to very low concentrations of lufenuron. First and second instar larvae fed higher concentrations of lufenuron

had normal growth and physical activity for several hours, but the insects died at about the time of the next ecdysis. Third instar larvae fed high concentrations of lufenuron underwent pupariation, but the puparia had an abnormal appearance. The anterior spiracles failed to evert. Thus, insect development is affected by lufenuron at all stages when chitin synthesis occurs. Another aspect of insect development affected by this compound was egg hatching even though oviposition was normal. The embryos completed development but failed to rupture the vitelline membrane. These results indicated that maternally derived lufenuron can affect egg hatching, which requires the use of chitinous mouth parts by the newly ecdysed larvae. The adults showed no mortality and had no flight disability even when fed high levels of lufenuron, indicating that once all chitin-containing structures had been formed, this "chitin inhibitor" had very little effect on adult morphology and function. However, the benzoylphenylureas may not be affecting CHS activity directly because diflubenzuron did not inhibit incorporation of UDP-GlcNAc into chitin microfibrils in an *in vitro* assay using a microsomal preparation from *T. castaneum* (Cohen and Casida, 1980). It is more likely that the benzoylphenylurea class of insecticides interferes with a step in the assembly of the cuticle and/or PM rather than chitin synthesis *per se*.

11.3.6.2. Genetics of chitin synthesis Several *Drosophila* genes involved in controlling cuticle morphology have been characterized (Jurgens *et al.*, 1984; Nusslein-Volhard *et al.*, 1984; Wiechaus *et al.*, 1984; Ostrowski *et al.*, 2002). These genes are *krotzkopf verkehrt* (*kkv*), *knickkopf* (*knk*), *grainy head (grh), retroactive (rtv)*, and *zepellin* (*zep*). All of these mutations result in poor cuticle integrity and reversal of embryo orientation in the egg to varying degrees. The homozygous mutant embryos failed to hatch. When these mutant embryos were mechanically devitellinized, the cuticles became grossly enlarged, yielding the "blimp" phenotype. Ostrowski *et al.* (2002) characterized the *kkv* gene and identified it as a *CHS*-like gene. Interestingly, embryos derived from wild-type females treated with high concentrations of lufenuron displayed a similar "blimp" phenotype when devitellinized, indicating that either genetic or chemical disruption of chitin deposition leads to this phenotype. The *knk* gene codes for a protein with sequence similarity to a protein component of the nuclear spindle matrix and is located on chromosome 3 close to the *kkv* gene near the centromere. The *knk* and *kkv* functions are not additive and *kkv* appears to be epistatic to *knk*, which is expressed at very low levels compared to the *kkv* gene as indicated by mRNA levels. The *knk* and *zep* genes appear to function in the epidermis prior to cuticle deposition because they exacerbate the effect of a heterozygous *shotgun* (*shg*) mutation, which codes for an E-cadherin-like protein. The *shg* gene is recessive, but in a *knk/knk* or *zep/zep* background, the cuticle is fragmented suggesting that the protein products of these genes interact with cadherin to reinforce the cuticle by promoting adhesion of the epithelia. Thus, products of all of the "blimp" class of genes, including *kkv*, control the integrity of the embryonic cuticle. It is also possible that some of these genes, whose functions have not been identified yet, may be involved directly or indirectly in extrusion or polymerization of chitin microfibrils. Alternatively, these proteins may reinforce chitin–chitin or chitin–protein interactions. For example, the *grh* gene encodes a GATA family transcription factor that regulates the expression of a DOPA decarboxylase needed for the production of precursors of cuticular protein cross-linking agents (Bray and Kafatos, 1991). It is also possible that some of these proteins are involved in vesicular trafficking and/or targeting CHS to plasma membrane plaques that are associated with chitin synthesis (Locke and Huie, 1979).

11.4. Chitin Degradation

Chitinases are among a group of proteins that insects use to digest the structural polysaccharide in their exoskeletons and gut linings during the molting process (Kramer *et al.*, 1985; Kramer and Koga, 1986; Kramer and Muthukrishnan, 1997; Fukamizo, 2000). Chitin is digested in the cuticle and PM to GlcNAc by a binary enzyme system composed of a chitinase (CHI) and a β-*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985; Filho *et al.*, 2002). The former enzyme from molting fluid hydrolyzes chitin into oligosaccharides, whereas the latter, which is also found in the molting fluid, further degrades the oligomers to the monomer from the nonreducing end. This system also probably operates in the gut during degradation of chitin in the PM or in digestion of chitin-containing prey.

Chitinase (EC 3.2.1.14, endochitinase) is defined as an enzyme that catalyzes the random hydrolysis of *N*-acetyl-β-D-glucosaminide β-1,4-linkages in chitin and chitodextrins. Chitinases are found in a variety of organisms besides insects including bacteria, fungi, plants, and marine and land animals (Watanabe and Kono, 2002). Many genes encoding chitinolytic enzymes including several from insects

(**Table 2**) have been cloned and characterized. Some chitinases are now being used for biotechnological applications in agriculture and healthcare (Patil *et al.*, 2000).

Chitinases are members of the superfamily of *O*-glycoside hydrolases, which hydrolyze the glycosidic bond in polysaccharides or between a sugar and a noncarbohydrate moiety. The International Union for Biochemistry and Molecular Biology enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally based on their molecular mechanism. Such a classification, however, does not reflect the structural features of these enzymes. Another classification of glycoside hydrolases into families is based on amino acid sequence similarities. This classification is expected to: (1) reflect the structural features of these enzymes better than their sole substrate specificity; (2) help to reveal the evolutionary relationships between these enzymes; and (3) provide a convenient tool to derive mechanistic information (Henrissat and Bairoch, 1996). There are 91 families of glycosylhydrolases and to date all mechanistically characterized insect chitinases belong to family 18 (Coutinho and Henrissat, 1999; CAZY, 2004). Unlike family 19 chitinases that are found almost exclusively in plants, members of family 18 have been found in a wide variety of sources including bacteria, yeasts and other fungi, nematodes, arthropods, and even vertebrates such as mice, chickens, and humans (Nagano *et al.*, 2002). The vertebrate proteins probably function as defensive proteins against chitin-containing pathogenic organisms.

11.4.1. Insect *N*-Acetylglucosaminidases

Beta-*N*-acetylglucosaminidases (EC 3.2.1.30) have been defined as enzymes that release β-*N*-acetylglucosamine residues from the nonreducing end of chitooligosaccharides and from glycoproteins with terminal *N*-acetylglucosamine. Insect β-*N*-acetylglucosaminidases are members of family 20 of the glycosylhydrolases (Coutinho and Henrissat, 1999; CAZY, 2004). These enzymes have been detected in the molting fluid, hemolymph, integument, and gut tissues of several species of insects (Kramer and Koga, 1986 and references therein). A β-*N*-acetylglucosaminidase also has been detected in the gut of *A. aegypti*, where its activity increased dramatically after blood feeding (Filho *et al.*, 2002). Beta-*N*-acetylglucosaminidases also hydrolyze synthetic substrates such as *p*-nitrophenyl *N*-acetylglucosamine and 4-methylumbelliferyl oligo-β-*N*-acetylglucosamines. These two substrates have proven to be very useful in assays of these enzymes.

During development, β-*N*-acetylglucosaminidase activities are the highest in hemolymph a few days prior to larval or pupal ecdysis and in molting fluid from pharate pupae (Kimura, 1976, 1977; Turner *et al.*, 1981). Two different enzymes with different physical and kinetic properties have been purified from the lepidopterans *B. mori* and *M. sexta*. The first enzyme (EI), which is found in larval and pharate pupal molting fluid and in pupal hemolymph, is probably involved in the turnover of chitobiose and possibly chitooligosaccharides because it has a lower K_m for these substrates than does the second (EII) enzyme. EII is found in larval and pupal hemolymph and has a lower K_m for pNpGlcNAc. The role of the enzyme (EII) is unclear, but its natural substrates may be glycoproteins containing terminal *N*-acetylglucosamines. However, this specificity remains to be proven.

11.4.2. Catalytic Mechanism of Insect *N*-Acetylglucosaminidases

N-acetylglucosaminidases have lower K_m values for substrates containing *N*-acetylglucosamine than those with *N*-acetylgalactosamine residues. They release monosaccharides from the nonreducing end by an exocleavage mechanism. Two ionizable groups with pKa values of 3.8 and 8.1 are involved in catalysis (Koga *et al.*, 1982). Studies with competitive inhibitors such as δ-lactone derivatives of *N*-acetylglucosamine and *N*-acetylgalactosamine suggested that the active site of enzyme EI consists of subsites that bind larger substrates than does the active site of the EII enzyme. EI has a lower K_m than EII for the chitooligosaccharides and a larger K_m for pNpβGlcNAc, properties that are consistent with the two enzymes having different endogenous substrate specificities.

11.4.3. Cloning of cDNAs for Insect *N*-Acetylglucosaminidases

cDNAs for epidermal β-*N*-acetylglucosaminidases of *B. mori* (GenBank accession no. S77548), *B. mandarina* (accession no. AAG48701), *T. ni* (accession no. AAL82580), and *M. sexta* (accession no. AY368703) have been isolated and characterized (Nagamatsu *et al.*, 1995; Zen *et al.*, 1996; Goo *et al.*, 1999). A search of the *Drosophila* and *Anopheles* genome databases also revealed the presence of closely related genes encoding β-*N*-acetylglucosaminidases. These genes encode closely related proteins (70-75% amino acid sequence identity between the *Manduca* and *Bombyx* enzymes) of approximately 68 kDa. The conceptual proteins contain leader peptides of 22-23 amino acids followed by stretches of

Table 2 Properties of insect chitinases

Species	*Common name*	*Tissue source*	*Number of amino acids*	*Domain structure*[a]	*GI no.*	*Reference*
Aedes aegypti	Yellow fever mosquito	ND	574	Cat-linker-ChBD 3ChBDs-3Cats	2564719	de la Vega *et al.* (1998)
Anopheles gambiae	Malaria mosquito	Gut	525	Cat-linker-ChBD	2654602	Shen and Jacobs-Lorena (1997)
Bombyx mori	Silkworm	Epidermis/gut	565	Cat-linker-ChBD	1841851, 10119784	Kim *et al.* (1998), Mikitani *et al.* (2000), Abdel-Banat and Koga (2001)
Chelonus sp. venom	Wasp	Venom gland	483	Cat-linker-ChBD	1079185	Krishnan *et al.* (1994)
Chironomus tentans	Midge	Cell line	475	Cat	2113832	Feix *et al.* (2000)
Choristoneura fumiferana	Spruce budworm	Epidermis/fat body	557	Cat-linker-ChBD	21913148	Zheng *et al.* (2002)
Drosophila melanogaster	Fruit fly	ND	508	Cat	17647257	de la Vega *et al.* (1998), Adams *et al.* (2000)
		ND	484	Cat	24655584	
		ND	458	ChBD-Cat	17647259	
Glossina morsitans	Tsetse fly	Fat body	460	Cat-ChBD	18201665	Yan *et al.* (2002)
Hyphantria cunea	Fall webworm	Epidermis	553	Cat-linker-ChBD	1841853	Kim *et al.* (1998)
Lutzomyia longipalpis	Sand fly	Midgut	474	Cat-linker-ChBD	28863959	Ramalho-Ortigão and Traub-Csekö (2003)
Manduca sexta	Tobacco hornworm	Epidermis/gut	554	Cat-linker-ChBD	1079015	Kramer *et al.* (1993), Choi *et al.* (1997)
Phaedon cochleariae	Mustard beetle	Gut	405	Cat	4210812	Girard and Jouanin (1999)
Spodoptera litura	Common cutworm	Epidermis	552	Cat-linker-ChBD	9971609	Shinoda *et al.* (2001)
Tenebrio molitor	Yellow mealworm	ND	2838	5 Cats+5 linkers+4 ChBDs+2 Mucs	21038943	Royer *et al.* (2002)

[a]Cat, catalytic domain; linker, linker region; ChBD, chitin-binding domain; Muc, mucin-like domain.
ND, not determined.

mature N-terminal amino acid sequences experimentally determined from *N*-acetylglucosaminidases purified from either the molting fluid or integument of these two species. The amino acid sequences include two regions that are highly conserved among *N*-acetylglucosaminidases from a variety sources including bacteria, yeast, mouse, and humans (Zen *et al.*, 1996). The *M. sexta* gene was expressed most abundantly in epidermal and gut tissues prior to metamorphosis and was induced by 20-hydroxyecdysone. The inductive effect of molting hormone was suppressed by juvenoids (Zen *et al.*, 1996).

11.4.4. Cloning of Genes Encoding Insect Chitinases

A chitinase from *M. sexta*, which is a 535 amino acid long glycoprotein (Chi535), as well as the cDNA and gene that encode it (*MsCHI*, accession no. AAC04924) were the first insect chitinase and gene to be isolated and characterized (Koga *et al.*, 1983; Kramer *et al.*, 1993; Choi *et al.*, 1997; Kramer and Muthukrishnan, 1997). They represent the most extensively studied chitinase enzyme–gene system in any insect species and they have become a model for study of other insect chitinases and their genes. Since the cloning of the *M. sexta* gene in 1993, cDNAs or genomic clones for several other insect chitinases have been isolated and sequenced (**Table 2**). The organization of most of these genes is very similar to that of *M. sexta* and most of the proteins display a domain architecture consisting of catalytic, linker, and/or chitin-binding domains similar to MsCHI. These genes/enzymes include epidermal chitinases from the silkworm, *B. mori* (Kim *et al.*, 1998; Abdel-Banat and Koga, 2001), the fall webworm, *Hyphantria cunea* (Kim *et al.*, 1998), wasp venom (*Chelonus* sp.) (Krishnan *et al.*, 1994), the common cutworm, *Spodoptera litura* (Shinoda *et al.*, 2001), a molt-associated chitinase from the spruce budworm, *Choristoneura fumiferana* (Zheng *et al.*, 2002), and midgut-associated chitinases from the malaria mosquito, *A. gambiae* (Shen and Jacobs-Lorena, 1997), yellow fever mosquito, *A. aegypti* (de la Vega *et al.*, 1998), the beetle *Phaedon cochleariae* (Girard and Jouanin, 1999), and the sand fly, *Lutzomyia longipalpis* (Ramalho-Ortigão and Traub-Csekö, 2003), and several deduced from the *Drosophila* genome data. A smaller linkerless fatbody-specific chitinase from the tsetse fly, *Glossina morsitans* (Yan *et al.*, 2002) and a very large epidermal chitinase with five copies of the catalytic-linker-chitin binding domain from the yellow mealworm, *Tenebrio molitor* (Royer *et al.*, 2002) have also been described.

Recently, a gene encoding another type of chitinase from the silkworm, *BmChi-h*, has been reported (Daimon *et al.*, 2003). The encoded chitinase shared extensive similarities with microbial and baculoviral chitinases (73% amino acid sequence identity to *Serratia marcescens* chitinase and 63% identity to *Autographa californica* nuclear polyhedrosis virus chitinase). Even though this enzyme had the signature sequence characteristic of family 18 chitinases, it had a rather low percentage of sequence identity with the family of insect chitinases listed in **Table 2**. It was suggested that an ancestral species of *B. mori* acquired this chitinase gene via horizontal gene transfer from *Serratia* or a baculovirus. Unlike the chitinases listed in **Table 2**, which typically have a leader peptide, catalytic domain, a serine/threonine(S/T)-rich domain and a C-terminal chitin-binding domain, BmChi-h chitinase has a leader peptide, one copy of module w1 domain that is found only in bacterial and baculoviral chitinases (Perrakis *et al.*, 1994; Henrissat, 1999), and a catalytic domain. Apparently, *B. mori* is not alone among insects possessing such a chitinase of bacterial origin. A protein in the molting fluid of *M. sexta*, which cross-reacted with an antibody to *M. sexta N*-acetylglucosaminidase, was found to have an N-terminal amino acid sequence closely resembling that of *Serratia* chitinase (Zen *et al.*, 1996). The N-terminal sequence of this protein was identical to that of BmChi-h up to the 25th amino acid residue, which strongly suggested that an ortholog of this chitinase gene exists in *M. sexta* as well. It will be interesting to investigate in the future whether this enzyme is widespread and found in other insect species. A search of the *Drosophila* and *Anopheles* genome databases, however, failed to identify any chitinase-like protein with an amino acid sequence identity to BmChi-h of greater than 40% (S. Muthukrishnan *et al.*, unpublished data).

Reports of multiple forms of insect chitinases, which can be generated by several mechanisms, have appeared. Some of these proteins are no doubt products of multiple genes as described in the previous paragraph. Others are likely the result of posttranslational modifications that are caused by glycosylation and/or proteolysis, which can lead to larger glycosylated forms and smaller truncated forms (Koga *et al.*, 1983; Wang *et al.*, 1996; Gopalakrishnan *et al.*, 1995; Arakane *et al.*, 2003). Another cause can be alternative splicing of mRNA. In *B. mori*, alternative splicing of the primary transcript from a single chitinase gene generates heterogeneity within the products (Abdel-Banat and Koga, 2002). Larger chitinase-like proteins have been observed in the mosquito *Anopheles* and

it has been proposed that these zymogenic proteins are activated via proteolysis by trypsin (Shen and Jacobs-Lorena, 1997). However, Filho *et al.* (2002) found no evidence for such activation in the mosquito *Aedes* because high levels of chitinase activity were observed early after a blood meal and even in the guts of unfed insects. Putative zymogenic forms have been reported in other insects as well (Koga *et al.*, 1992; Bhatnagar *et al.*, 2003). However, the existence of a chitinase zymogen is still speculative in most cases because all of the fully characterized cDNAs encoding full-length insect chitinases apparently have the mature catalytic domains immediately following their leader peptides and there is no indication of the presence of pre-proproteins (**Table 2**). Preliminary evidence suggests that most, if not all, of the larger proteins reacting with chitinase antibodies are multimeric forms that are enzymatically inactive and produced as a result of intermolecular disulfide pairing. These larger forms appear after long periods of storage of the monomeric enzyme and they can be reconverted to enzymatically active monomeric forms by treatment with thiol reagents (Y. Arakane *et al.*, unpublished data).

11.4.5. Modular Structure of Insect Chitinases

A multidomain structural organization is generally observed in polysaccharide-degrading enzymes where one or more domains are responsible for hydrolysis and other domains are responsible for associating with the solid polysaccharide substrate. In addition, there usually are linker regions between the two types of domains, which also may be responsible, at least in part, for some functional properties of the enzymes. For example, the first chitinases shown to contain catalytic, linker, and chitin-binding or fibronectin-like domains were isolated from the bacterium *Bacillus circulans* (Watanabe *et al.*, 1990), the yeast *S. cereviseae* (Kuranda and Robbins, 1991), and the parasitic nematode *B. malayi* (Venegas *et al.*, 1996). Insect chitinases possess a similar structural organization, as do some other nematode, microbial, and plant chitinases as well as fungal cellulases. Observed in all of these enzymes is a multidomain architecture that may include a signal peptide and one or more of the following domains: catalytic domains, cysteine-rich chitin-binding domains, fibronectin-like domains, mucin-like domains, and S/T-rich linker domains, with the latter usually being rather heavily glycosylated (Tellam, 1996; Henrissat, 1999; Suzuki *et al.*, 1999). For example, chitinases from the bacterium *S. marcescens*, fall into three classes with sizes ranging from 36 to 52 kDa, which are composed of different combinations of catalytic domains, fibronectin type-III-like domains, and N- or C-terminal chitin-binding domains (Suzuki *et al.*, 1999). A novel multidomain structure exhibited by an insect chitinase is that of the yellow mealworm beetle, *T. molitor* (Royer *et al.*, 2002). This protein is unusually large, with a calculated molecular mass of approximately 320 kDa. It contains five catalytic domains, five S/T-rich linker domains, four chitin-binding domains, and two mucin-like domains. Gene duplication and domain deletion mechanisms have probably generated the diversity and multiplicity of chitinase genes in insects, as was demonstrated previously in bacteria (Saito *et al.*, 2003).

The structure of the catalytic domain of insect chitinase is a $(\beta\alpha)_8$ TIM (triose phosphate isomerase) barrel fold, which is one of the most common folds found in proteins (Nagano *et al.*, 2001, 2002). During protein evolution, domain shuffling has allowed this fold to acquire a large number of specific catalytic functions such as enzymes with a glycosidase activity like insect chitinase. The presence of additional domains such as linker and chitin-binding domains appears to further enhance the catalytic properties of these enzymes.

Figure 4 shows a phylogenetic tree of 16 insect chitinases inferred from an amino acid sequence alignment. All five of the lepidopteran enzymes and only one dipteran chitinase reside in the upper portion of the tree, whereas the other seven dipteran, one hymenopteran, and two coleopteran enzymes appear in the lower part.

Manduca sexta CHI is much smaller than the *Tenebrio* enzyme and much less complex in domain structure with only a single N-terminal catalytic domain (376 amino acids long), a linker domain (about 100 amino acids long), and a C-terminal chitin-binding domain (ChBD, 58 amino acids long) (Arakane *et al.*, 2003). Alternate domain arrangements occur in other glycosylhydrolases. For example, class I, class IV, and class VII plant chitinases contain an N-terminal ChBD and a G/P-rich linker preceding the catalytic domain (Raikhel *et al.*, 1993; Neuhaus, 1999), whereas fungal cellulases, like insect chitinase, possess a threonine/serine/proline-rich linker between the N-terminal catalytic domain and the C-terminal cellulose-binding domain (Srisodsuk *et al.*, 1993). The *Manduca* CHI linker region that is rich in T and S residues is also rich in P, D, and E residues, which qualifies it as a PEST sequence-containing protein according to Rogers *et al.* (1986). That composition suggested that insect chitinase might be rapidly degraded via the intracellular ubiquitin-conjugating enzymes/proteosome system, which recognizes the PEST

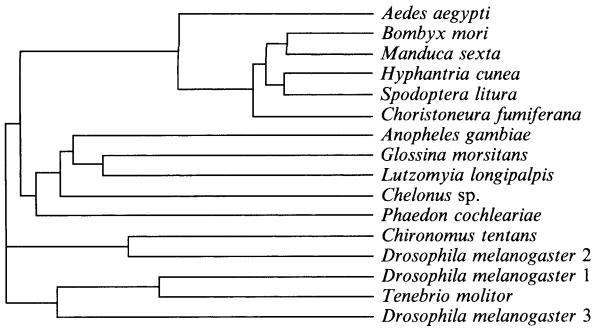


Figure 4 Phylogenetic tree of insect chitinases inferred from an amino acid sequence alignment of 16 enzymes from *Aedes aegypti*, *Bombyx mori*, *Manduca sexta*, *Hyphantria cunea*, *Spodoptera litura*, *Choristoneura fumiferana*, *Anopheles gambiae*, *Glossina morsitans*, *Lutzomyia longipalpis*, *Chelonus* sp., *Phaedon cochleariae*, *Chironomus tentans*, *Drosophila melanogaster*, and *Tenebrio molitor*. The GI numbers are listed in **Table 2**. Multiple sequence alignment was performed using Clustal W software (Thompson *et al.*, 1994) and the tree was built using the neighbor-joining method (Saitou and Nei, 1987).

sequence so that proteosomes can digest the conjugated protein when it is localized intracellularly. However, since insect chitinase is a secreted protein, it would be exposed to intracellular proteases or the ubiquitin-conjugating system only for a relatively short period of time. Instead, the linker apparently helps to optimize interactions with the insoluble substrates and to stabilize proteins, and perhaps also helps to protect protease-susceptible bonds in the catalytic domains from hydrolysis. Recombinant chitinases that contain this linker region were more stable in the presence of midgut digestive proteases than recombinant proteins lacking the linker region (Arakane *et al.*, 2003). The linker domain also may have another function involving protein trafficking. Recombinant forms of *Manduca* CHI lacking amino acid residues beyond position 376 accumulated intracellularly during expression in the baculovirus-insect cell line, whereas all of the forms that had an additional ten amino acids or longer stretches of the linker domain were secreted into the media (Arakane *et al.*, 2003). We concluded, therefore, that for secretion of recombinant protein to the outside of the insect cells to occur, the N-terminal portion of the linker region (residues 377–386) must be present, in addition to the 19 amino acid long N-terminal leader peptide. For secretion, the linker region may also need to be O-glycosylated because when glycosylation was inhibited by the addition of tunicamycin, insect chitinase accumulated intracellularly in an insect cell line (Gopalakrishnan *et al.*, 1995). Some of the critical residues for secretion/glycosylation, therefore, may involve residues between amino acids 376 and 386 (which includes two threonines) because the truncated Chi376 accumulated intracellularly, whereas Chi386 was secreted. Site-directed mutagenesis of these residues might help to answer the question about what residues in the linker region are required for secretion.

Peptides linking protein domains are very common in nature and some, unlike the insect chitinase linker, are believed to join domains rather passively without disturbing their function or affecting their susceptibility to cleavage by host proteases (Argos, 1990; Gilkes *et al.*, 1991). Linker peptides with G, T, or S residues are most common, perhaps because those residues are relatively small with G providing flexibility and T and S being uncharged but polar enough to interact with solvent or by their ability to hydrogen bond to water or to the protein backbone to achieve conformational and energetic stability. The interdomain linker peptide of a fungal cellobiohydrolase apparently has a dual role in providing the necessary distance between the two functional domains and also facilitating the dynamic adsorption process led by the cellulose-binding domain (Srisodsuk *et al.*, 1993). Solution conformation studies of a fungal cellulase with two domains revealed that its linker exhibited an extended conformation leading to maximum distance between the two domains and that heterogeneous glycosylation of the linker was likely a key factor defining its extended conformation (Receveur *et al.*, 2002). Since the domain structure of *M. sexta* CHI is similar to that of this fungal cellulase, these two enzymes may have similar global structural characteristics. Circular dichroism (CD) spectra of the wild-type and truncated insect chitinases were consistent with the hypothesis that whereas the catalytic and ChBDs possess secondary structure, the linker region itself does not (Arakane *et al.*, 2003).

Mammalian chitinase is similar in structure to *M. sexta* chitinase in both the catalytic domain and ChBD, but it lacks a linker domain (Tjoelker *et al.*, 2000). The absence of the ChBD does not affect the ability of the human enzyme to hydrolyze soluble oligosaccharides but does abolish hydrolysis of the insoluble substrate, a result consistent with the hypothesis that the function of the ChBD is to facilitate heterogeneous catalysis on insoluble substrates. One of the basic functions of carbohydrate-binding domains (CBD) is thought to be to help localize the enzyme on the insoluble substrate to enhance the efficiency of degradation (Linder and Teeri, 1997). These domains aid in recognition and hydrolysis of substrates that can exist in several physical states, i.e., contain both crystalline and noncrystalline forms. In general, for many glycosylhydrolases,

the binding specificity of the carbohydrate-binding domain mirrors that of the catalytic domain and these two domains are usually in relatively close association. Such is not the case for *Manduca* CHI, which has a very long linker of over several hundred angstroms.

Like their cognate catalytic domains, CBDs are classified into families of related amino acid sequences. The ChBD of insect chitinases belongs to carbohydrate-binding module family 14, which consists of approximately 70 residues (Coutinho and Henrissat, 1999; CAZY, 2004). Only three subfamilies of chitin-binding modules have been identified to date and the ChBD of *M. sexta* CHI is a member of subfamily 1 (Henrissat, 1999). Such a carbohydrate-binding function has been demonstrated in several other carbohydrolases and carbohydrate-binding proteins. Other CBD families, family 17 and family 28, both of which recognize cellulose, have been found to act in a cooperative manner either by modifying the action of the catalytic module or by targeting the enzyme to areas of cellulose that differ in susceptibility to hydrolysis (Boraston *et al.*, 2003). ChBDs may play a similar role in chitinases. These domains are attached not only to catalytic domains but also to chitinase-like proteins devoid of enzyme activity. The ChBDs can be either N- or C-terminal and may be present as a single copy or as multiple repeats. They are cysteine-rich and have several highly conserved aromatic residues (Shen and Jacobs-Lorena, 1999). The cysteine residues help to maintain protein folding by forming disulfide bridges and the aromatic residues interact with saccharides in the ligand-binding pocket. The PM proteins, mucins, which have affinity for chitin, also have a six-cysteine-containing peritrophin-A/mucin consensus sequence that is similar to ChBD sequences in chitinases (Tellam *et al.*, 1999; Morlais and Severson, 2001).

When fused with the catalytic domain of *M. sexta* CHI, both insect and rice ChBDs promoted the binding to and hydrolysis of chitin (Arakane *et al.*, 2003). The influence of extra substrate-binding domains has been examined previously using a fungal chitinase that was constructed to include plant and fungal carbohydrate-binding domains (Limón *et al.*, 2001). The addition of those domains increased the substrate-binding capacity and specific activity of the enzyme toward insoluble substrates of high molecular mass such as ground chitin or chitin-rich fungal cell walls. On the other hand, removal or addition of cellulose-binding domains can reduce or enhance, respectively, the ability of cellulases to degrade crystalline cellulose (Chhabra and Kelly, 2002). When a second cellulose-binding domain was fused to *Trichoderma reesei* cellulase, the resulting protein had a much higher affinity for cellulose than the protein with only a single binding domain (Linder *et al.*, 1996). Likewise, the *M. sexta* CHI catalytic domain fused with two ChBDs associated with chitin more strongly than any of the single ChBD-containing proteins or the protein devoid of a ChBD (Arakane *et al.*, 2003). This domain apparently helps to target the secreted enzyme to its insoluble substrate.

The chitin-binding domain of insect chitinase not only has the function of associating with insoluble chitin, but it may also help to direct the chitin chain into the active site of the catalytic domain in a manner similar to the processive hydrolysis mechanism proposed for *S. marcescens* chitinase A (ChiA), which has a very short ChBD (Uchiyama *et al.*, 2001). However, whether such an extended linker like that of *M. sexta* chitinase can direct the substrate into the active site in a manner similar to that proposed for a shorter linker is unknown. Catalytically, the full-length *M. sexta* CHI was two- to fourfold more active in hydrolyzing insoluble colloidal chitin than any of the other truncated enzymes with an intact catalytic domain, but all of the enzymes were comparable in turnover rate when two soluble substrates, carboxymethyl-chitin-remazol-brilliant-violet (CM-chitin-RBV), which is a chromogenic chitin derivative that is O-carboxymethylated, and MU-$(GlcNAc)_3$, a fluorogenic oligosaccharide substrate, were hydrolyzed (Arakane *et al.*, 2003). A moderate increase in catalytic efficiency of hydrolysis of insoluble substrate was observed when the catalytic domain was fused with the ChBD. When the C-terminal ChBD was deleted from a bacterial chitinase (*Aeromonas caviae*), this truncated chitinase was active also, but it liberated longer oligosaccharide products than did the full-length enzyme (Zhou *et al.*, 2002). Thus, as was observed with other carbohydrolases such as xylanases (Gill *et al.*, 1999), the ChBD of insect chitinase facilitates hydrolysis of insoluble, but not soluble, substrates, and also influences the size of the oligosaccharide products generated. The linker region also can influence the functionality of the carbohydrate-binding domain. When a fungal cellulose-binding domain was fused with a fungal S/T-rich linker peptide, the fusion protein adsorbed to both crystalline and amorphous cellulose. However, deletion of the linker peptide caused a decrease in cellulose adsorption and a higher sensitivity to protease digestion (Quentin *et al.*, 2002). The addition of a carbohydrate-binding module to a catalytic domain via a linker domain may increase the catalytic efficiency for degradation of the insoluble

polysaccharide and may modify the finely tuned binding specificity of the enzyme (McLean *et al.*, 2002; Lehtio *et al.*, 2003).

Figure 5 shows a theoretical model structure for *M. sexta* chitinase that is complexed with chitin oligosaccharides in both the catalytic domain and ChBD at a time subsequent to hydrolysis of a larger oligosaccharide. What is perhaps most striking is the very long linker (>200 Å) between the other domains. Apparently, the enzyme is tethered to the cuticle by the ChBD, which anchors the catalytic domain to the insoluble substrate and localizes the hydrolysis of chitin to an area with a radius of several hundred angstroms. The use of such a tethered enzyme would help to prevent diffusion of the soluble enzyme from the insoluble polysaccharide. In the case of *Tenebrio* chitinase, which consists of five catalytic, five linker, and six chitin-binding domains (Royer *et al.*, 2002), one could envision a situation where a much wider area of the chitin–protein matrix undergoes intensive degradation by a much larger tethered enzyme.

11.4.6. Mechanism of Catalysis

Insect chitinases are members of family 18 of the glycosylhydrolases (CAZY, 2004), which generally utilize a mechanism where retention of the anomeric configuration of the sugar donor occurs via a substrate-assisted catalysis, rather than a mechanism similar to lysozyme, which involves a proton donor and an electrostatic stabilizer (Fukamizo, 2000). However, a recent kinetic study of bacterial family 18 chitinases demonstrated that substrates lacking the *N*-acetyl group and thus incapable of anchimeric assistance were nevertheless hydrolyzed, suggesting that the reaction mechanism of family 18 chitinases cannot be fully explained by the substrate-assisted catalysis model (Honda *et al.*, 2003). Therefore, additional studies are still required to understand fully the reaction mechanism of family 18 chitinases.

The interaction of insect chitinases with insoluble chitin in the exoskeleton and PM is rather complex and believed to be a dynamic process that involves adsorption via a substrate-binding domain, hydrolysis, desorption, and repositioning of the catalytic domain on the surface of the substrate. This degradative process apparently requires a coordinated action of multiple domains by a mechanism that is not well understood. In addition to the catalytic events, the mechanism of binding of the enzyme onto the heterogeneous surface of native chitin is poorly characterized. Hydrolysis of chitin to GlcNAc is accomplished by a binary enzyme system composed of a chitinase and a β-*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985;

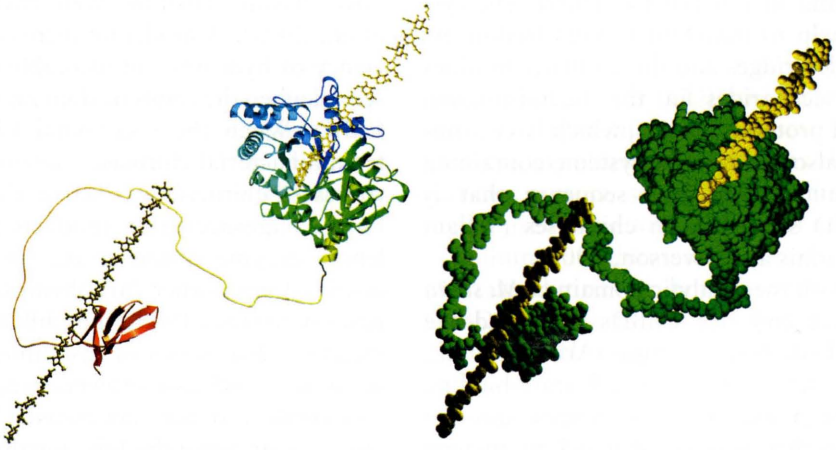


Figure 5 Ribbon (left) and space-filling (right) model structures of *Manduca sexta* chitinase with the catalytic and chitin-binding domains shown in complexes with chitin oligosaccharides (yellow). In the ribbon representation, the polypeptide chain is color-coded, beginning with blue at the N-terminus and proceeding through the rainbow to red at the C-terminus. The catalytic domain structure (top) was modeled using the program SOD (Kleywegt *et al.*, 2001) with human chitotriosidase (PDB entry code 1LG1) (Fusetti *et al.*, 2002) serving as the template. The chitin-binding domain (bottom) was similarly obtained using tachychitin (PDB entry 1DQC) (Suetake *et al.*, 2000) as the template. The linker region is shown as a random coil as predicted by secondary structure prediction software and supported by circular dichroism data. The oligosaccharides are shown as stick models (left) and space-filling models (right). Substrate binding to the catalytic domain was modeled using the available structures of complexes from glycosyl hydrolase family 18, while binding to the chitin-binding domain was modeled based on sequence conservation within the subfamily. *M. sexta* chitinase is a glycoprotein that is glycosylated especially in the linker region; however, no carbohydrate is shown in the model. (The model was constructed by Wimal Ubhayasekera and Dr. Sherry Mowbray, Swedish University of Agricultural Sciences, Uppsala.)

Filho *et al.*, 2002). The former enzyme hydrolyzes the insoluble polymer into soluble oligosaccharides, whereas the latter further degrades the oligomers to the monomer from the nonreducing end. Mechanistically, chitinases of family 18 hydrolyze chitin with retention of the anomeric configuration at the cleavage site, involving a double-displacement mechanism where a substrate-assisted catalysis occurs (Tomme *et al.*, 1995; Henrissat, 1999; Zechel and Withers, 2000; Brameld *et al.*, 2002). *B. mori* chitinase utilizes a retaining mechanism, yielding products that retain the β-anomeric configuration (Abdel-Banat *et al.*, 1999). All of the enzymes of this family are inhibited by allosamidin, a transition state analog inhibitor which apparently is diagnostic for enzymes that utilize the retaining mechanism (Koga *et al.*, 1987; Bortone *et al.*, 2002; Brameld *et al.*, 2002; Lu *et al.*, 2002). Analysis of the products from the hydrolysis of chitin oligosaccharides by the family 18 chitinase from *S. marcescens* revealed variable subsite binding preferences, anomeric selectivity, and the importance of individual binding sites for the processing of short oligosaccharides compared to the cumulative recognition and processive hydrolysis mechanism used to digest the polysaccharide (Aronson *et al.*, 2003).

Polysaccharide-hydrolyzing enzymes are known to exhibit nonideal kinetic behavior because they often are susceptible to inhibition by both substrates and products (Väljamäe *et al.*, 2001). All insect chitinases examined were found to be susceptible to inhibition by oligosaccharide substrates but to varying extents (Fukamizo and Kramer, 1985; Fukamizo *et al.*, 1995; Fukamizo, 2000). Apparently, the oligosaccharide substrate molecules can bind to these enzymes in such a manner that none of the target bonds is properly exposed to the functional groups of catalytic amino acids or the substrate may bind in only noncatalytic subsites of the larger active site, forming nonproductive instead of productive complexes. Cellulose is also degraded by the synergistic action of cellulolytic enzymes, which also display this characteristic substrate inhibition (Väljamäe *et al.*, 2001). Site-directed mutagenesis studies involving amino acids present in the putative catalytic site of *M. sexta* CHI have identified residues required for catalysis (Huang *et al.*, 2000; Lu *et al.*, 2002; Zhang *et al.*, 2002). Aspartic acids 142 and 144, tryptophan 145, and glutamic acid 146 were identified as residues very important for catalysis and also for extending the pH range of enzyme activity into the alkaline pH range. Acidic and aromatic residues in other family 18 chitinases also are important for substrate binding and catalysis (Watanabe *et al.*, 1993, 1994; Uchiyama *et al.*, 2001; Bortone *et al.*, 2002). Some of these residues are essential only for crystalline chitin hydrolysis, whereas others are important not only for crystalline chitin hydrolysis but for other substrates as well (Watanabe *et al.*, 2003).

11.4.7. Glycosylation of Insect Chitinases

Manduca sexta CHI is moderately *N*-glycosylated in the catalytic domain and heavily *O*-glycosylated in the linker region (Arakane *et al.*, 2003). The insect cell line TN-5B1-4 (Hi 5), which is routinely used for expression of recombinant foreign glycoprotein, synthesizes proteins with both *N*- and *O*-linked oligosaccharides (Davidson *et al.*, 1990; Davis and Wood, 1995; Jarvis and Finn, 1995; Hsu *et al.*, 1997). Results of experiments investigating the effects of the *N*-glycosylation inhibitor tunicamycin on recombinant expression of insect chitinases in these cells indicated that the proteins were glycosylated prior to being secreted by the cells (Gopalakrishnan *et al.*, 1995; Zheng *et al.*, 2002). Direct chemical and enzymatic analyses confirmed that *M. sexta* CHI was both *N*- and *O*-glycosylated. Prolonged deglycosylation with a mixture of *N*- and *O*-glycosidases resulted in a protein that was smaller by about 6 kDa accounting for about 30 sugar residues per mole of protein (Arakane *et al.*, 2003). Because *N*-linked oligosaccharides in insects typically have six or seven residues, two of which are GlcNAc (Paulson, 1989; Kubelka *et al.*, 1995), the best estimate of the distribution of *N*-glycosylation indicated a single or possibly two sites of *N*-glycosylation in the catalytic domain and *O*-glycosylation of between 10 and 20 serine or threonine residues in the linker region. *O*-glycosylation may involve mainly addition of galactose and *N*-acetylgalactosamine.

The chitinase from *B. mori* also is probably glycosylated because this protein and its breakdown product (65 kDa) stain with periodic acid–Schiff reagent. Further, the apparent mobility of the protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 88 kDa, whereas the molecular weight of the mature protein predicted from the cDNA sequence is only 60 kDa (Koga *et al.*, 1997). This protein has an S/T-rich linker similar to the *M. sexta* chitinase. On the other hand, the chitinase from wasp venom which has only a short linker region and is low in serine and threonine has nearly the same molecular weight as the one predicted from the cDNA sequence, suggesting that this protein may not be glycosylated (Krishnan *et al.*, 1994). Thus, there is a good

correlation between the presence of an S/T-rich linker and extensive glycosylation (predominantly O-glycosylation) of the chitinolytic proteins.

Glycosylation of the linker region may help to prevent proteolytic cleavage(s) at sites between the catalytic and chitin-binding domains. Such a functional role of glycosylated regions has been observed in some bacterial cellulases (Langsford *et al.*, 1987). The full-length and near full-length O-glycosylated forms of *M. sexta* CHI were the most stable proteins when incubated with the midgut proteinases of the hornworm (Arakane *et al.*, 2003). Protein modeling studies using the crystal structures of other family 18 glycosylhydrolases as templates suggested that the catalytic domain of *M. sexta* CHI has a $(\beta\alpha)_8$-triose phosphate isomerase (TIM) barrel structure (Kramer and Muthukrishnan, 1997; Nagano *et al.*, 2002). The ChBD probably exhibits a multistranded β-sheet structure based on similarity to tachycitin (Suetake *et al.*, 2000). We know of no structures computed or proposed for linker domains, which may be very hydrophilic and rather flexible as well as potentially susceptible to proteolytic degradation unless they are protected by glycosylation. The CD spectrum of the linker domain was consistent with the lack of any secondary structure in this domain (**Figure 5**). It is conceivable that during the developmental period of maximum chitinase activity, the enzyme is fully glycosylated. When required, a glycosidase(s) could be produced that would remove sugar residues, thus exposing several more peptide bonds for proteolytic cleavage. Alternatively, proteolytic cleavage may be reduced because of glycosylation. Consistent with this notion is the finding that analysis of molting fluid from *M. sexta* and *B. mori* revealed the presence of truncated forms of catalytically active chitinases with sizes ranging from 50 to 60 kDa (Kramer and Koga, 1986; Koga *et al.*, 1997; Abdel-Banat *et al.*, 1999). We also detected similar truncated forms in our insect cell recombinant chitinase expression system, especially several days subsequent to infection with the recombinant baculovirus (Gopalakrishnan *et al.*, 1995).

11.4.8. Antigenicity of Insect Chitinases

Invertebrate chitinases have been reported to elicit allergies in mammals. For example, a high prevalence of IgE antibodies to a tick chitinase was identified in canine atopic dermatitis with the chitinase formally designated Der f 15 (McCall *et al.*, 2001). In ticks, this chitinase was localized in the proventriculus and intestine, indicating that it has a digestive, rather than molting-related, function. Like insect chitinase, tick chitinase is extensively O-glycosylated on multiple sites along the 84 amino acid long S/T-rich sequence in the molecule. The transmission blocking antibody MF1 from the blood of gerbils infected with the nematode *B. malayi* was found to be directed against a microfilarial chitinase (Fuhrman *et al.*, 1992). This antibody mediates the clearance of peripheral microfileremia in gerbils, indicating that chitinase is indeed a potent antigen. Even though it is unclear which region of the nematode chitinase is highly antigenic, the most probable one is the S/T–rich region known to be O-glycosylated.

The primary epitope recognized by antibodies elicited by *Manduca* chitinases is the highly glycosylated S/T-rich linker region (Arakane *et al.*, 2003). Other highly immunogenic insect proteins that also are extensively O-glycosylated in S/T-rich domains similar to the linker region of *Manduca* CHI are peritrophins-55 and -95 from the sheep blowfly, *L. cuprina* (Tellam *et al.*, 2000, 2003). The sera of sheep vaccinated with these peritrophins exhibited a strong immune response that also inhibited growth of blowfly larvae (Casu *et al.*, 1997; Tellam *et al.*, 2003).

11.4.9. Other Possible Enzymes of Chitin Metabolism

Chitin deacetylases and chitosanases are two other enzymes that play major roles in chitin catabolism in other types of organisms. Chitin deacetylase catalyzes the removal of acetyl groups from chitin. This enzyme is widely distributed in microorganisms and may have a role in cell wall biosynthesis and in counteracting plant defenses (Tsigos *et al.*, 2000). There is one report of an insect chitin deacetylase in physogastric queens of the termite *Macrotermetes estherae* (Sundara Rajulu *et al.*, 1982). However, there have been no follow-up studies about this enzyme in other insect species. To our knowledge, there are no reports of chitosanases present in insects.

11.5. Nonenzymatic Proteins That Bind to Chitin

There are approximately 32 families of CBDs that are defined as contiguous amino acid sequences within a carbohydrate-active enzyme or noncatalytic analogs, which exhibit a discrete fold having carbohydrate-binding activity (CAZY, 2004). One or more members in families 1, 2, 3, 5, 12, 14, 16, 18, and 19 are reported to bind chitin. Most, if not all, of the insect ChBDs, however, belong only to family 14.

Several chitinase-related proteins have been identified in insects, which are catalytically inactive because they are missing an amino acid residue critical for hydrolytic activity but nonetheless are carbohydrate-binding proteins with either a single copy or multiple repeats of ChBDs. These proteins may act as growth factors or play a defensive function as anti-inflammatory proteins. A chitinase homolog glycoprotein HAIP (hemolymph aggregation inhibiting protein) occurs in hemolymph of the lepidopteran *M. sexta*, which inhibits hemocyte aggregation (Kanost *et al.*, 1994). A similar immunoreactive protein was detected in hemolymph of three other lepidopterans, *B. mori*, *Heliothis zea*, and *Galleria mellonella*. These proteins may have a role in modulating adhesion of hemocytes during defensive responses. Another glycoprotein, Ds47, which is produced *in vitro* by a *Drosophila* embryo-derived cell line and by fat body and hemocytes, may play a role in promoting the growth of imaginal discs (Kirkpatrick *et al.*, 1995; Bryant, 2001). Another chitinase-related protein is induced together with a chitinase and β-*N*-acetylglucosaminidase by ecdysteroid in the anterior silk gland of *B. mori* at molting and at metamorphosis (Takahashi *et al.*, 2002). The former is rather large in size and has a novel structure consisting of tandemly repeated catalytic domain-like plus linker sequences, but it has only one ChBD located in the middle of the protein. All of these proteins are evolutionarily related to chitinases, but they apparently have acquired a new growth-promoting or infection-resistance function that does not require catalytic activity. Evidently, chitinases have evolved into these lectin-like proteins by mutation of key residues in the active site, which abolishes enzyme activity and fine tunes the ligand-binding specificity.

Chitin-binding proteins in vertebrates, invertebrates, and plants share a common structural motif composed of one to eight disulfide bonds and several aromatic residues, apparently the result of convergent evolution (Shen and Jacobs-Lorena, 1999; Suetake *et al.*, 2000). A chitin-binding antifungal peptide from the coconut rhinoceros beetle, *Oryctes rhinoceros*, scarabaecin, is only 36 residues in length and contains only one disulfide bond (Hemmi *et al.*, 2003). It shares significant tertiary structural similarity with ChBDs of other invertebrates and plants that have multiple disulfide bonds, even though there is no overall sequence similarity. Other invertebrate proteins that contain one or more ChBDs include the peritrophins (Tellam *et al.*, 1999), mucins (Casu *et al.*, 1997; Wang and Granados, 1997; Tellam *et al.*, 1999; Rayms-Keller *et al.*, 2000; Sarauer *et al.*, 2003), and tachycitin (Suetake *et al.*, 2000).

Other proteins that bind to chitin include several lectins and cuticular proteins (see **Chapter 12**). The lectins are related to ChBDs found in PM and chitinases. Many insect cuticular proteins contain an amino acid sequence motif of approximately 35 residues known as the R&R consensus sequence (Rebers and Willis, 2001). This sequence, however, has no similarity to the cysteine-rich ChBDs found in chitinases, some PM proteins, and lectins. There are no or very few cysteine residues in the cuticular protein ChBDs (noncysChBD). Thus, there are two distinct classes of invertebrate ChBDs, those with the chitin-binding domain found in lectins, chitinases, and PM proteins (cysChBDs) and those with the cuticular protein chitin-binding domain (noncysChBDs). Homology modeling of insect cuticle proteins using the bovine plasma retinol binding protein as a template predicted an antiparallel β-sheet half-barrel structure as the basic folding motif where an almost flat surface consisting of aromatic amino acid side chains interacts with the polysaccharide chains of chitin (Hamodrakas *et al.*, 2002).

In mammals there are several nonenzymatic members of the chitinase protein family. Oviduct-specific glycoprotein (OGP), a member of this family, is believed to be involved in the process of fertilization such as sperm function and gamete interactions (Araki *et al.*, 2003). However, OGP was not essential for *in vitro* fertilization in mice, and so the functionality of OGP remains unknown. The human cartilage protein HCgp-39 is a chitin-specific lectin (Renkema *et al.*, 1998; Houston *et al.*, 2003) that is overexpressed in articular chondrocytes and certain cancers. It is thought to be an anti-inflammatory-response protein and/or to play a role in connective tissue remodeling. In contrast to chitinases, which bind and hydrolyze chitin oligosaccharides but do not undergo large conformational changes, HCgp-39 exhibits a large conformational change upon ligand binding, which appears to signal the presence of chitinous pathogens such as fungi and nematodes (van Aalten, 2003). The murine *Ym1* gene belongs to a family of mammalian genes encoding nonenzymatic proteins that are homologous to the chitinases from lower organisms, such as insects, nematodes, bacteria, and plants (Sun *et al.*, 2001). YKL-40 is a nonenzymatic member of the mammalian family 18 glycosylhydrolases, which is a growth factor for connective tissue cells and stimulates migration of endothelial cells (Johansen *et al.*, 2003). It is secreted in large amounts by

human osteosarcoma cells and murine mammary tumors, and it is also elevated in patients with metastatic breast cancer and colorectal cancer. These homologous mammalian proteins have no demonstrable chitinase activity and, therefore, cannot be considered chitinases. The biological functions of these proteins remain obscure. However, these proteins likely function through binding to carbohydrate polymers and since they are secreted from activated hemocytes, they may have a function in immunity such as a hemocyte inhibition (Falcone *et al.*, 2001). Sequence comparison of these nonenzymatic and enzymatic proteins indicates that the enzymatic proteins have evolved into these lectins by the mutation of key residues in the active site and optimization of the substrate-binding specificity (Fusetti *et al.*, 2002).

11.6. Regulation of Chitin Degradation

The *M. sexta* chitinase and *N*-acetylglucosaminidase genes were shown to be upregulated by ecdysteroid (see **Chapter 7**) and down-regulated by the juvenile hormone mimic (see **Chapter 8**), phenoxycarb, in larval abdomens cut off from their hormonal sources (Fukamizo and Kramer, 1987; Koga *et al.*, 1991; Kramer *et al.*, 1993; Zen *et al.*, 1996). Differential display was used to show that chitinase expression was regulated not only by ecdysteroid but also by juvenile hormone in the beetle *T. molitor* (Royer *et al.*, 2002). Northern blot analysis of RNA from epidermis and 20-hydroxyecdysone-injected pupae showed that chitinase transcripts were correlated with molting hormone levels during metamorphosis. In addition, topical application of a juvenile hormone (JH) analog indirectly induced expression of chitinase mRNA. Thus, the *Tenebrio* chitinase gene is an early direct ecdysteroid-responsive one at the transcriptional level, but unlike *M. sexta* chitinase, it is apparently a direct target of JH as well. In the former case, the level at which JH regulates chitinase mRNA levels remains to be determined. The 20-hydroxyecdysone agonist, tebufenozide, induced expression of *C. fumiferana* chitinase when it was injected into mature larvae. The enzyme was produced 24 h post treatment in both the epidermis and molting fluid (Zheng *et al.*, 2003).

11.7. Chitin Metabolism and Insect Control

Chitinases have been used in a variety of ways for insect control and other purposes (Kramer *et al.*, 1997; Gooday, 1999). Several chitinase inhibitors with biological activity have been identified based on natural products chemistry (Spindler and Spindler-Barth, 1999), such as allosamidin (Rao *et al.*, 2003) which mimics the carbohydrate substrate, and cyclic peptides (Houston *et al.*, 2002). Although useful for biochemical studies, none of these chitin catabolism inhibitors have been developed for commercial use primarily because of their high cost of production and potential side effects. As we learn more details about chitinase catalysis, it might become more economically feasible to develop and optimize chitinase inhibitors for insect pest management.

Additional uncharacterized steps in chitin synthesis and/or assembly of chitin microfibrils, on the other hand, have proved to be important for developing control chemicals that act selectively on economically important groups of insect pests (Verloop and Ferrell, 1977; Ishaaya, 2001). The benzoylphenylureas have been developed as commercial compounds for controlling agricultural pests. These antimolting insecticides are relatively nontoxic to mammals due to their strong protein binding and extensive metabolization to less toxic compounds (Bayoumi *et al.*, 2003). Studies using imaginal discs and cell-free systems indicated that benzoylphenylureas inhibit ecdysteroid-dependent GlcNAc incorporation into chitin (Mikolajczyk *et al.*, 1994; Oberlander and Silhacek, 1998). Those results suggest that benzoylphenylureas affect ecdysone-dependent sites, which leads to chitin inhibition. However, the site of action of the benzoylphenylureas still is not well known. Recently, several heteryl nucleoside nonhydrolyzable transition state analogs of UDP-GlcNAc were synthesized and evaluated for fungicidal activity, but they were not assayed for insecticidal activity (Behr *et al.*, 2003).

Entomopathogens secrete a plethora of extracellular proteins with potential activity in insect hosts. One of these proteins is chitinase, which is used by fungi such as *Metarhizium anisopliae* to help penetrate the host cuticle and render host tissues suitable for consumption (St. Leger *et al.*, 1996; Krieger de Moraes *et al.*, 2003). Among the 10 most frequent transcripts in a strain of *M. anisopliae* are three encoding chitinases and one a chitosanase, presumably reflecting a greater propensity to produce chitinases for host cuticle penetration (Freimoser *et al.*, 2003a). Expressed sequence tag analysis of *M. anisopliae* may hasten gene discovery to enhance development of improved mycoinsecticides. However, when *M. anisopliae* was transformed to overexpress its native chitinase, the pathogenicity to the tobacco hornworm was unaltered, suggesting that

wild-type levels of chitinase are not limiting for cuticle penetration (Screen *et al.*, 2001). Another fungal species, *Conidiobolus coronatus*, also produces both endo- and exo-acting chitinolytic enzymes during growth on insect cuticle (Freimoser *et al.*, 2003b). Apparently, both *M. anisopliae* and *C. coronatus* produce a chitinolytic enzyme system to degrade cuticular components.

Both microbial and insect chitinases have been shown to enhance the toxicity of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) (Regev *et al.*, 1996; Tantimavanich *et al.*, 1997; Ding *et al.*, 1998; Sampson and Gooday, 1998; Wiwat *et al.*, 2000). For example, when the chitinolytic activities of several strains of *B. thuringiensis* were compared with their insecticidal activity, it was determined that the enzyme could enhance the toxicity of Bt to *Spodoptera exigua* larvae by more than twofold (Liu *et al.*, 2002). Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic microorganisms (review: Kramer *et al.*, 1997). Synergistic effects between chitinolytic enzymes and microbial insecticides have been reported as early as the 1970s. Bacterial chitinolytic enzymes were first used to enhance the activity of Bt and a baculovirus. Larvae of *C. fumiferana* died more rapidly when exposed to chitinase–Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff and Valero, 1972; Morris, 1976; Lysenko, 1976). Mortality of gypsy moth, *Lymantria dispar*, larvae was enhanced when chitinase was mixed with Bt relative to a treatment with Bt alone in laboratory experiments (Dubois, 1977). The toxic effect was correlated positively with enzyme levels (Gunner *et al.*, 1985). The larvicidal activity of a nuclear polyhedrosis virus toward *L. dispar* larvae was increased about fivefold when it was administered with a bacterial chitinase (Shapiro *et al.*, 1987). Chitin synthesis-inhibiting antifungal agents such as flufenoxuron and nikkomycin were used to promote the infection of silkworms with *B. mori* nucleopolyhedrovirus (Arakawa, 2002, 2003; Arakawa and Sugiyama, 2002; Arakawa *et al.*, 2002). The mechanism of viral infection enhancement by these agents is not established, but it may involve destruction of PM structure, which would facilitate tissue invasion.

Inducible chitinolytic enzymes from bacteria cause insect mortality under certain conditions. These enzymes may compromise the structural integrity of the PM barrier and improve the effectiveness of Bt toxin by enhancing contact of the toxin molecules with their epithelial membrane receptors. For example, five chitinolytic bacterial strains isolated from midguts of *Spodoptera littoralis* induced a synergistic increase in larval mortality when combined with Bt spore-crystal suspensions relative to either an individual bacterial strain or a Bt suspension alone (Sneh *et al.*, 1983). An enhanced toxic effect toward *S. littoralis* also resulted when a combination of low levels of a truncated recombinant Bt toxin and a bacterial endochitinase was incorporated into a semisynthetic insect diet (Regev *et al.*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* toward diamondback moth larvae (Wiwat *et al.*, 1996). Liu *et al.* (2002) recently reported that several strains of Bt produced their own chitinases, which had synergistic larvicidal activity with the endotoxins.

In biopesticide development research, we used a family 18 insect chitinase as an enhancer protein for baculovirus toxicity and as a host plant resistance factor in transgenic plants. Introduction of an insect chitinase cDNA into *A. californica* multiple nuclear polyhedrosis viral (AcMNPV) DNA accelerated the rate of killing of fall armyworm compared to the wild-type virus (Gopalakrishnan *et al.*, 1995). Baculoviral chitinases themselves play a role in liquefaction of insect hosts (Hawtin *et al.*, 1997; Thomas *et al.*, 2000). A constitutively expressed exochitinase from *B. thuringiensis* potentiated the insecticidal effect of the vegetative insecticidal protein Vip when they were fed to neonate larvae of *S. litura* (Arora *et al.*, 2003). Some granuloviruses, on the other hand, do not utilize chitinases in a similar manner, which helps to explain why some granulovirus-infected insects do not lyse at the end of the infection process (Wormleaton *et al.*, 2003). Mutagenesis of the AcMNPV chitinase gene resulted in cessation of liquefaction of infected *T. ni* larvae, supporting a role of chitinase in virus spread (Thomas *et al.*, 2000). However, the insecticidal activity of insect chitinase was not substantial enough for commercial development. We have attempted with little success to improve the catalytic efficiency and stability of this enzyme so that its pesticidal activity would be enhanced (Lu *et al.*, 2002; Zhang *et al.*, 2002; Arakane *et al.*, 2003). Nevertheless, tobacco budworms were killed when reared on transgenic tobacco expressing a truncated, enzymatically active form of insect chitinase (Ding *et al.*, 1998). We also discovered a synergistic interaction between insect chitinase expressed in transgenic tobacco plants and Bt (applied as a spray at sublethal levels) using the tobacco hornworm as the test insect. In contrast to results obtained with the tobacco budworm, studies with the hornworm revealed no consistent differences in larval growth or foliar damage when the insects were reared on

first-generation transgenic chitinase-positive tobacco plants as compared to chitinase-negative control plants. When Bt toxin was applied at levels where no growth inhibition was observed on control plants, chitinase-positive plants had significantly less foliar damage and lower larval biomass production. These results indicated that the insect chitinase transgene did potentiate the effect of sublethal doses of Bt toxin and vice versa (Ding *et al.*, 1998). Tomato plants have been transformed with fungal chitinase genes with concomitant enhancement in resistance to insect pests (Gongora *et al.*, 2001). Effects observed include reduced growth rates and increased mortality, as well as a decrease in plant height and flowering time with an increase in the number of flowers and fruits (Gongora and Broadway, 2002). Chitinase-secreting bacteria have been used to suppress herbivorous insect pests. A chitinase gene-transformed strain of *Enterobacter cloacae* digested the chitinous membranes of phytophagous ladybird beetles, *Epilachna vigintioctopunctata*, and also suppressed leaf-feeding and oviposition when the beetles ingested transformed bacteria entrapped in alginate microbeads sprayed on tomato seedlings (Otsu *et al.*, 2003).

Several GlcNAc-specific lectins from plants have been evaluated for insect toxicity (Harper *et al.*, 1998; Macedo *et al.*, 2003). These proteins appear to disrupt the integrity of the PM by binding to chitin or glycan receptors on the surface of cells lining the insect gut. They also may bind to glycosylated digestive enzymes and inhibit their activity. Another type of plant chitin-binding protein is the seed storage protein, vicilin, which is actually a family of oligomeric proteins with variable degrees of glycosylation (Macedo *et al.*, 1993; Shutov *et al.*, 1995). Some vicilins are insecticidal to bruchid beetles and stalk borers (Sales *et al.*, 2001; Mota *et al.*, 2003). Apparently, these proteins bind to the PM, causing developmental abnormalities and reduced survival rates. To date no carbohydrate-binding protein derived from an insect has been evaluated for biocidal activity. A novel approach has been proposed to develop strategies for insect control by utilizing chitin-binding molecules to specifically target formation of the PM. Calcofluor, a chemical whitener with chitin-binding properties, was used as a model compound in the diet to inhibit PM formation in *T. ni* and also to increase larval susceptibility to baculovirus infection (Wang and Granados, 2000b). It also was effective in suppressing PM formation in *Spodoptera frugiperda* and at the same time in preventing the establishment of a decreasing gradient of proteinases along the midgut tissue (Bolognesi *et al.*, 2001).

Another type of hydrolytic enzyme with a ChBD has been shown to exhibit insecticidal activity in plants. Maize accumulates a 33 kDa cysteine protease containing a ChBD in response to insect feeding (Perchan *et al.*, 2002). This enzyme apparently damages the insect's PM by utilizing the ChBD to localize itself at the chitin-protein-rich PM, where the PM proteins are digested, rendering the PM dysfunctional. Another protease with a chitin-binding domain has been described from *A. gambiae*, which may be involved in insect defense (Danielli *et al.*, 2000). This 147 kDa protein, sp22D, is expressed in a variety of tissues, most strongly in hemocytes, and is secreted into the hemolymph. Upon bacterial infection, the transcripts for this protein increase by about twofold suggesting a role in insect defense. This protein has a multidomain organization that includes two copies of an N-terminal ChBD, a C-terminal protease domain, and additional receptor domains. It binds strongly to chitin and undergoes complex proteolytic processing during pupal to adult metamorphosis. It has been proposed that exposure of this protease to chitin may regulate its activity during tissue remodeling or wounding.

Recently, two synthetic peptides were found to inhibit *A. gambiae* midgut chitinase and also to block sporogonic development of the human malaria parasite, *Plasmodium falciparum*, and avian malaria parasite, *P. gallinaceum*, when the peptides were fed to infected mosquitoes (Bhatnagar *et al.*, 2003). The design of these peptides was based on the putative proregion sequence of mosquito midgut chitinase. The results indicated that expression of chitinase inhibitory peptides in transgenic mosquitoes might alter the vectorial capacity of mosquitoes to transmit malaria.

11.8. Concluding Remarks

Although chitin was discovered nearly two centuries ago, it remains a biomaterial in waiting because, unlike other natural materials such as collagen and hyaluronic acid, very few technological uses have been developed (Khor, 2002; Tharanathan and Kittur, 2003). There are many unanswered questions about chitin morphology and chitin deposition in the insect cuticle and PM. We do not know how or whether chitin forms covalent interactions with other components in these extracellular matrices. Chitosan, on the other hand, does react with quinones (Muzzarelli and Muzzarelli, 2002; Muzzarelli *et al.*, 2003). Thus, if there were any free amino groups in insect chitin, C–N linkages between chitin and catechols would be expected (Schaefer *et al.*,

1987). We do not yet understand how factors such as metal ions affect chitin metabolism. In fungi, ions such as zinc were found to alter chitin deposition and morphology (Lanfranco *et al.*, 2002). Perhaps, in insects there is an ionic effect on differential expression of CHS isozymes.

We know much more about insect chitinolytic enzymes than about insect chitin biosynthetic enzymes. Many questions remain about the biosynthesis of insect chitin, not the least of which are why insects have multiple genes for CHS, how many CHSs are required to make an insect, at what developmental stages are the various CHSs produced, and what are the unique properties and functions of each CHS. Of particular interest is the role of alternate splicing in generating different isoforms of CHSs from the same gene. The developmental cues that control alternate splicing and how they affect chitin synthesis and/or deposition will be subjects of future studies. The cloning of CHS genes should soon lead to availability of large amounts of recombinant enzymes or subdomains thereof using appropriate expression systems. Studies with pure proteins and the availability of molecular probes will provide a better understanding of the chitin biosynthetic pathway and its regulation in the future.

Two other major questions about insect chitin biosynthesis are: what is the mechanism of the initiation phase and is there an autocatalytic initiator. Like glycogen synthesis, chitin synthesis probably includes both initiation and elongation phases. As the initiator of glycogen synthesis, glycogenin transfers glucose from UDP-glucose to itself to form an oligosaccharide–protein primer for elongation (Gibbons *et al.*, 2002). Like chitin synthase, glycogenin is a glycosyltransferase, which raises the question of whether chitin synthase has an autocatalytic function similar to glycogenin and whether there is a chitinogenin-like protein. Another possibility is the participation of a lipid primer for chitin synthesis. Recently, cellulose synthesis in plants was found to involve the transfer of lipid-linked cellodextrins to a growing glucan chain (Read and Bacic, 2002). The lipid in this case was sitosterol-β-glucoside.

Little is known about the catalytic mechanism of any insect CHS. Once insect CHS-related recombinant proteins are obtained, site-directed mutagenesis can be used to probe for essential residues in the catalytic and regulatory domains. It is likely that acidic amino acids play critical roles in CHS catalysis in a manner comparable to those identified in other glycosyltransferases (Hefner and Stockigt, 2003) and in yeast chitin synthases (Nagahashi *et al.*, 1995).

Chitinolytic enzymes are gaining importance for their biotechnological applications in agriculture and healthcare (Patil *et al.*, 2000). Additional success in using chitinases for different applications depends on a better understanding of their biochemistry and regulation so that their useful properties can be optimized through genetic and biochemical engineering. Reasons for the rather high multiplicity of domain structures for insect and other chitinases are not fully understood. So far little success has occurred in using chitinase in pest control applications, but it may prove more useful as an enhancer protein in a cocktail with other biopesticides targeted at the cuticle or gut. Also, only a few catalytic domains or chitin-binding domains or various combinations thereof have been evaluated for biocidal activity and thus, further toxicological experimentation is warranted.

Although substantial progress in studies of insect chitin metabolism has occurred since the first edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* was published in 1985, we still do not know much about how chitin is produced and transported across the membrane so that it can interact perfectly with other components for assembly of the supramolecular extracellular structures called the exoskeleton and PM. These materials are still very much biochemical puzzles in which we do not understand well how the various components come together during morphogenesis or are digested apart during the molting process. Hopefully, this chapter will stimulate more effort to understand how insects utilize chitin metabolism for growth and development, and to develop materials that may perturb insect chitin metabolism for pest management purposes.

Acknowledgments

The authors are grateful to Sherry Mowbray, Wimal Ubhayasekera, Yasuyuki Arakane, Qingsong Zhu, David Hogenkamp, Renata Bolognesi, Tamo Fukamizo, Daizo Koga, Walter Terra and Clelia Terra for their help with the preparation and/or comments on various aspects of this review. Supported in part by National Science Foundation grant no. 0316963. Mention of a proprietary product does not constitute a recommendation or endorsement by the US Department of Agriculture. The US Department of Agriculture is an equal opportunity/affirmative action employer and all agency services are available without discrimination. This is contribution no. 04-058-C of the Kansas Agricultural Experiment Station.

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